

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C07K 14/705, C12N 5/10, 15/09, 15/11, 15/12, 15/62, G01N 33/566</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/40114</b> <b>(43) International Publication Date:</b> 12 August 1999 (12.08.99)
<b>(21) International Application Number:</b> PCT/US99/02361 <b>(22) International Filing Date:</b> 3 February 1999 (03.02.99)  <b>(30) Priority Data:</b> 60/073,767 5 February 1998 (05.02.98) US  <b>(71) Applicants (for all designated States except US):</b> MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). MERCK FROSST CANADA & CO. [CA/CA]; 16711 Trans-Canada Highway, Kirkland, Quebec H9H 3L1 (CA). UNIVERSITY OF TEXAS HEALTH SCIENCE CENTER AT SAN ANTONIO [US/US]; 7703 Floyd Curl Drive, San Antonio, TX 78284 (US). NATIONAL INSTITUTES OF HEALTH [US/US]; 36 Convent Drive, Bethesda, MD 20892-4094 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> LIU, Quingyun [CN/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). McDONALD, Terrence [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). BONNERT, Timothy, P. [GB/GB]; Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR (GB). NG, Gordon, Yiu, Kon [CA/CA]; 16711 Trans-Canada Highway, Kirkland, Quebec H9H 3L1 (CA). KOLAKOWSKI, Lee, F., Jr. [US/US]; 7703 Floyd		<b>(74) Common Representative:</b> MERCK & CO., INC.; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).  <b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> NOVEL GABA <sub>B</sub> RECEPTOR DNA SEQUENCES  <b>(57) Abstract</b>  DNA encoding a novel human GABA <sub>B</sub> receptor subunit, HG20, as well as the protein encoded by the DNA, is provided. Also provided is DNA encoding a novel murine GABA <sub>B</sub> receptor subunit, GABA <sub>B</sub> R1a, as well as the protein encoded by the DNA. Heterodimers of HG20 protein and GABA <sub>B</sub> R1a protein that form a functional GABA <sub>B</sub> receptor are disclosed. Methods of identifying agonists and antagonists of the GABA <sub>B</sub> receptor are also provided.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						



TITLE OF THE INVENTION  
NOVEL GABA<sub>B</sub> RECEPTOR DNA SEQUENCES

CROSS-REFERENCE TO RELATED APPLICATIONS

5 Not applicable.

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not applicable.

10 REFERENCE TO MICROFICHE APPENDIX

Not applicable.

FIELD OF THE INVENTION

15 The present invention is directed to a novel human DNA sequence encoding HG20, a subunit of the GABA<sub>B</sub> receptor, the protein encoded by the DNA, and uses thereof. The present invention also is directed to the murine GABA<sub>B</sub>R1a subunit of the GABA<sub>B</sub> receptor as well as to methods of combining an HG20 subunit with a GABA<sub>B</sub>R1a subunit to form a GABA<sub>B</sub> receptor having functional activity.

20

BACKGROUND OF THE INVENTION

Amino acids such as glutamic acid,  $\gamma$ -amino-butyric acid (GABA), and glycine are neurotransmitters that bind to specific receptors in the vertebrate nervous system and mediate synaptic transmission. Of these amino acids, GABA is the most widely distributed amino acid inhibitory neurotransmitter in the vertebrate central nervous system. The biological activities of GABA are mediated by three types of GABA receptors: ionotropic GABA<sub>A</sub> receptors, metabotropic GABA<sub>B</sub> receptors, and ionotropic GABA<sub>C</sub> receptors. Each type of receptor has its own characteristic molecular structure, pattern of gene expression, agonist and antagonist mediated pharmacological effects, and spectrum of physiological activities.

30 GABA<sub>A</sub> receptors mediate fast synaptic inhibition. They are heterooligomeric proteins (most likely pentamers) containing  $\alpha$ ,  $\beta$ ,  $\gamma$ , and perhaps  $\delta$ , subunits that function as ligand-gated Cl<sup>-</sup> channels and have binding sites for benzodiazepines, barbiturates, and neuroactive steroids. Bicuculline is a widely used antagonist of GABA<sub>A</sub> receptors.

Bicuculline is selective for GABA<sub>A</sub> receptors in that it has no effect on GABA<sub>B</sub> or GABA<sub>C</sub> receptors. The expression of GABA<sub>A</sub> receptors has been observed in a variety of brain structures (see. *e.g.*, McKernan & Whiting, 1996, Trends Neurosci. 16:139-143; Sequier et al., 1988, Proc. Natl. Acad. Sci. USA 85:7815-7819).

GABA<sub>C</sub> receptors are ligand-gated Cl<sup>-</sup> channels found in the vertebrate retina. They can be distinguished from GABA<sub>A</sub> and GABA<sub>B</sub> receptors in that they are insensitive to the GABA<sub>A</sub> receptor antagonist bicuculline and the GABA<sub>B</sub> receptor agonist (-)baclofen but are selectively activated by *cis*-4-aminocrotonic acid. GABA<sub>C</sub> receptors are composed of homooligomers of a category of GABA receptor subunits known as "ρ" subunits, the best-studied of which are ρ1 and ρ2. ρ1 and ρ2 share 74% amino acid sequence identity but are only about 30-38% identical in amino acid sequence when compared to GABA<sub>A</sub> receptor subunits. For a review of GABA<sub>C</sub> receptors, see Bormann & Feigenspan, 1995, Trends Neurosci. 18:515-518.

GABA<sub>B</sub> receptors play a role in the mediation of late inhibitory postsynaptic potentials (IPSPs). GABA<sub>B</sub> receptors belong to the superfamily of seven transmembrane-spanning G-protein coupled receptors that are coupled through G-proteins to neuronal K<sup>+</sup> or Ca<sup>++</sup> channels. GABA<sub>B</sub> receptors are coupled through G-proteins to neuronal K<sup>+</sup> or Ca<sup>++</sup> channels, and receptor activation increases K<sup>+</sup> or decreases Ca<sup>++</sup> conductance and also inhibits or potentiates stimulated adenylyl cyclase activity. The expression of GABA<sub>B</sub> receptors is widely distributed in the mammalian brain (*e.g.*, frontal cortex, cerebellar molecular layer, interpeduncular nucleus) and has been observed in many peripheral organs as well.

A large number of pharmacological activities have been attributed to GABA<sub>B</sub> receptor activation, *e.g.*, analgesia; hypothermia; catatonia; hypotension; reduction of memory consolidation and retention; and stimulation of insulin, growth hormone, and glucagon release (see Bowery, 1989, Trends Pharmacol. Sci. 10:401-407, for a review.) It is well accepted that GABA<sub>B</sub> receptor agonists and antagonists are pharmacologically useful. For example, the GABA<sub>B</sub> receptor agonist (-)baclofen, a structural analog of GABA, is a clinically effective muscle relaxant (Bowery & Pratt, 1992, Arzneim.-Forsch./Drug

Res. 42:215-223). (-)baclofen, as part of a racemic mixture with (+)baclofen, has been sold in the United States as a muscle relaxant under the name LIORESAL® since 1972.

GABAB receptors represent a large family of related proteins, new family members of which are still being discovered. For example, Kaupmann et al., 1997, Nature 386:239-246 (Kaupmann) reported the cloning and expression of two members of the rat GABAB receptor family, GABABR1a and GABABR1b. A variety of experiments using known agonists and antagonists of GABAB receptors seemed to indicate that GABABR1a and GABABR1b represented rat GABAB receptors. This conclusion was based primarily on the ability of GABABR1a and GABABR1b to bind agonists and antagonist of GABAB receptors with the expected rank order, based upon studies of rat cerebral cortex GABAB receptors. However, there were data that did not fit the theory that Kaupmann had cloned the pharmacologically and functionally active GABAB receptor. For example, Kaupmann noted that agonists had significantly lower binding affinity to recombinant GABABR1a and GABABR1b as opposed to native GABAB receptors. Also, Couve et al., 1998, J. Biol. Chem. 273:26361-26367 showed that recombinantly expressed GABABR1a and GABABR1b failed to target correctly to the plasma membrane and failed to give rise to functional GABAB receptors when expressed in a variety of cell types.

Examination of the amino acid and gene sequence of GABABR1a led Kaupmann to propose a structure for GABABR1a similar to that of the metabotropic glutamate receptor gene family. The metabotropic glutamate receptor family comprises eight glutamate binding receptors and five calcium sensing receptors which exhibit a signal peptide sequence followed by a large N-terminal domain believed to represent the ligand binding pocket that precedes seven transmembrane spanning domains. The hallmark seven transmembrane spanning domains are typical of G-protein coupled receptors (GPCRs), although metabotropic glutamate receptors and GABABR1a are considerably larger than most GPCRs and contain a signal peptide sequence. No significant amino acid sequence similarities were found between GABABR1a and GABAA receptors, GABAC receptors, or other typical GPCRs.

Despite work such as that of Kaupmann, pharmacological and physiological evidence indicates that a large number of amino acid binding GABA<sub>B</sub> receptors remain to be cloned and expressed in recombinant systems where agonists and antagonists can be efficiently  
5 identified. In particular, it would be extremely valuable to be able to recombinantly express GABA<sub>B</sub> receptors in such a manner that not only pharmacologically relevant ligand binding properties would be exhibited by the recombinant receptors, but also such that the recombinant receptors would show proper functional activity.

10

#### SUMMARY OF THE INVENTION

The present invention is directed to a novel human DNA that encodes a GABA<sub>B</sub> receptor subunit, HG20. The DNA encoding HG20 is substantially free from other nucleic acids and has the  
15 nucleotide sequence shown in SEQ.ID.NO.:1. Also provided is an HG20 protein encoded by the novel DNA sequence. The HG20 protein is substantially free from other proteins and has the amino acid sequence shown in SEQ.ID.NO.:2. Methods of expressing HG20 in recombinant systems and of identifying agonists and antagonists of HG20 are  
20 provided.

The present invention is also directed to a novel murine DNA that encodes a GABA<sub>B</sub> receptor subunit, GABA<sub>B</sub>R1a. The DNA encoding GABA<sub>B</sub>R1a is substantially free from other nucleic acids and has the nucleotide sequence shown in SEQ.ID.NO.:19. Also provided is  
25 a GABA<sub>B</sub>R1a protein encoded by the novel DNA sequence. The GABA<sub>B</sub>R1a protein is substantially free from other proteins and has the amino acid sequence shown in SEQ.ID.NO.:20. Methods of expressing GABA<sub>B</sub>R1a in recombinant systems and of identifying agonists and antagonists of HG20 are provided.

Also provided by the present invention are methods of co-expressing HG20 and GABA<sub>B</sub>R1a in the same cells. Such co-expression results in the production of a GABA<sub>B</sub> receptor that exhibits expected functional properties of GABA<sub>B</sub> receptors as well as expected ligand binding properties. Recombinant cells co-expressing HG20 and  
35 GABA<sub>B</sub>R1a are provided as well as methods of utilizing such recombinant cells to identify agonists and antagonists of GABA<sub>B</sub> receptors.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-B shows the complete cDNA sequence of HG20 (SEQ.ID.NO.:1).

5           Figure 2 shows the complete amino acid sequence of HG20 (SEQ.ID.NO.:2).

Figure 3A-B shows predicted signal peptide cleavage sites of HG20. All sequences shown are portions of SEQ.ID.NO.:2.

10           Figure 4 shows *in situ* analysis of the expression of HG20 RNA in squirrel monkey brain.

Figure 5A shows *in vitro* coupled transcription/translation of a chimeric FLAG epitope-HG20 (amino acids 52-941) protein.

15           Figure 5B shows the expression in COS-7 cells and melanophores of a chimeric FLAG epitope-HG20 (amino acids 52-941) protein.

Figure 6 shows a comparison of the amino acid sequences of a portion of the N-terminus of HG20 protein and the ligand binding domain of the *Pseudomonas aeruginosa* amino acid binding protein LIVAT-BP (Swiss Protein database accession number P21175). The upper sequence shown is from HG20 and corresponds to amino acids 63-259 of SEQ.ID.NO.:2. The lower sequence shown is from *Pseudomonas aeruginosa* LIVAT-BP and is SEQ.ID.NO.:16.

25           Figure 7 shows expression in mammalian cells of a chimeric HG20 protein.

Figure 8 shows a comparison of the amino acid sequences of HG20 and GABABR1b. The HG20 sequence is SEQ.ID.NO.:2. The GABABR1b sequence is SEQ.ID.NO.:17.

30           Figure 9 shows the expression of recombinant GABABR1a and HG20 in COS-7 cells. Lanes 1 and 2 show [<sup>125</sup>I]CGP71872 photolabeling of recombinant murine GABABR1a monomer and dimer in the presence (+) and absence (-) of 1  $\mu$ M unlabeled CGP71872. Lanes 3 and 4 show that GABABR1a antibodies 1713.1-1713.2 confirmed (+) expression of recombinantly expressed murine GABABR1a (referred to as mgb1a here) and absence (-) in pcDNA3.1 mock transfected cells.

35           Lanes 5 and 6 show [<sup>125</sup>I]CGP71872 photolabeling of human FLAG-HG20 in the presence (+) and absence (-) of 1  $\mu$ M unlabeled CGP71872. Lanes 7 and 8 show that an anti-FLAG antibody confirmed (+) the

expression of FLAG-HG20 (referred to as FLAG-gb2 here) and its absence (-) in pcDNA3.1 mock transfected cells. Experimental details were as in Examples 7-9 and 20 except that COS-7 rather than COS-1 cells were used.

5                Figure 10 shows co-localization of mRNA for HG20 and GABABR1a by *in situ* hybridization histochemistry in rat parietal cortex. Adjacent coronal sections of rat brain showing parietal cortex hybridized with radiolabelled GABABR1a (A) and HG20 (B) probes. Rat GABABR1a and HG20 probes were labelled using <sup>35</sup>S-UTP (A, B, and  
10                D), and autoradiograms were developed after 4 weeks. For co-localization studies, the rat GABABR1a probe was digoxigenin labelled and developed using anti-digoxigenin HRP, the TSA amplification method and biotinyl tyramide followed by streptavidin-conjugated CY3 (C). (D) shows autoradiography of the same field as in (C), denoting  
15                hybridization to HG20 mRNA. (E) is an overlay of images (C) and (D). Arrows denote some of the double-labelled cells. Scale bar = 0.5 mm in (A) and (B); scale bar = 50  $\mu$ m in (C-E).

                 Figure 11 shows functional complementation following co-expression of GABABR1a and HG20 in *Xenopus* melanophores. GABA  
20                mediated a dose-dependent aggregation response in melanophores co-expressing murine GABABR1a and FLAG-HG20 (■) that could be blocked with 100 nM (▼) and 1  $\mu$ M CGP71872 (▲). The response of GABA on mock-transfected cells is shown (●) as well as a control cannabinoid receptor subtype 2 response to HU210 ligand (inset). This experiment is  
25                representative of n=4.

                 Figure 12 shows GABAB receptor modulation of forskolin-stimulated cAMP synthesis in HEK293 cells. HEK293 cells stably expressing HG20 (hgb2-42) or GABABR1a (rgb1a-50) were transiently transfected with GABABR1a and HG20 expression plasmids to examine  
30                the effect of receptor co-expression on modulation of cAMP synthesis. All transfected cells were tested with 300  $\mu$ M baclofen or GABA (with 100  $\mu$ M AOAA and 100  $\mu$ M nipecotic acid) in the absence of forskolin and 30  $\mu$ M baclofen or GABA in the presence of 10  $\mu$ M forskolin. Wild-type HEK293 cells were tested with 250  $\mu$ M baclofen or 250  $\mu$ M GABA in the  
35                presence of 10  $\mu$ M forskolin. Data are presented as the percent of total cAMP synthesized in the presence of forskolin only. The data presented are from single representative experiments that have been replicated

twice. Fsk, forskolin; B, baclofen; G, GABA with AOAA and nipecotic acid. The two right-most set of bar graphs (labeled "B + Fsk" and "G + Fsk") show that in cells expressing both GABA<sub>B</sub>R1a and HG20 (rgb1a-50/hgb2 cells (□) and hgb2-42/rgb1a cells (■)), baclofen and GABA were  
5 able to mediate significant reductions in cAMP levels.

Figure 13 shows that co-expression of GABA<sub>B</sub>R1a and HG20 permits inwardly rectifying potassium channel (GIRK or Kir) activation in *Xenopus* oocytes. (A) Representative current families of Kir 3.1/3.2. Currents were evoked by 500 msec voltage commands from a  
10 holding potential of -10 mV, delivered in 20 mV increments from -140 to 60 mV. (B) In a protocol designed to measure the effects of various receptors on Kir currents, oocytes were held at -80 mV (a potential where significant inward current is measured). Expression of GABA<sub>B</sub>R1a or HG20 alone (with or without G $\alpha$ 1) resulted in no  
15 modulation of current after GABA treatment. Co-expression of GABA<sub>B</sub>R1a and FLAG-HG20 receptors followed by treatment with 100  $\mu$ M GABA resulted in stimulation of Kir 3.1/3.2. Shown are representative traces from at least three independent experiments under each condition.

20 Figure 14 shows immunoblotting of murine GABA<sub>B</sub>R1a and FLAG-HG20 transiently expressed in COS-7 cells. Digitonin-solubilized and anti-FLAG antibody immunoprecipitated membrane proteins were immunoblotted following SDS-PAGE with GABA<sub>B</sub>R1a antibodies 1713.1-1713.2. The conditions are as follows: mock pcDNA3.1  
25 vector transfected cells (lane 1), FLAG-HG20 expressing cells (lane 2), murine GABA<sub>B</sub>R1a expressing cells (lane 3), and cells coexpressing murine GABA<sub>B</sub>R1a and FLAG-HG20 (lane 4). The immunoreactive band corresponding to the GABA<sub>B</sub>R1a /HG20 heterodimer as well as a band corresponding to the predicted GABA<sub>B</sub>R1a monomer are denoted  
30 by arrows.

Figure 15 shows the complete cDNA sequence of murine GABA<sub>B</sub>R1a (SEQ.ID.NO.:19). The sequence shown has been deposited in GenBank (accession number AF114168).

35 Figure 16 shows the complete amino acid sequence of murine GABA<sub>B</sub>R1a (SEQ.ID.NO.:20). The sequence shown has been deposited in GenBank (accession number AF114168).

Figure 17A-B shows the results of experiments with N- and C-terminal fragments of murine GABABR1a. Figure 17A shows the results of coupled *in vitro* transcription/translation reactions; lane 1 = blank; lane 2 = full-length GABABR1a; lane 3 = N-terminal fragment of GABABR1a; lane 4 = C-terminal fragment of GABABR1a. Figure 17B shows the results of [<sup>125</sup>I]CGP71872 photoaffinity labeling; lane 1 = N-terminal fragment of GABABR1a; lane 2 = N-terminal fragment of GABABR1a in the presence of GABA; lane 3 = C-terminal fragment of GABABR1a; lane 4 = C-terminal fragment of GABABR1a in the presence of GABA.

Figure 18A-B shows the amino acid sequence (Figure 18A) (SEQ.ID.NO.:21) and nucleotide sequence (Figure 18B) (SEQ.ID.NO.:22) (GenBank accession number AJ012185) of a human GABABR1a.

Figure 19A-B shows the nucleotide sequence (SEQ.ID.NO.:23) (GenBank accession number Y11044) of a human GABABR1a.

Figure 20 shows a framework map of chromosome 9. The locations of the HG20 gene (referred to as "GPR 51"), markers, and the HSN-1 locus are indicated.

Figure 21 shows a hydropathy plot for murine GABABR1a.

Figure 22 shows a family tree of genes related to HG20. Abbreviations are as follows: hGB1a = human GABABR1a; mGB1a = mouse GABABR1a; rGB1a = rat GABABR1a; hGB1b = human GABABR1b; rGB1b = rat GABABR1b; ceGB1b = a *C. elegans* gene related to mammalian GABABR1a and GABABR1b; hGB2 = human HG20; ceGB2 = a *C. elegans* gene related to human HG20; MGRDROME = a metabotropic glutamate receptor from *Drosophila melanogaster*; MGR2 HUMAN = human metabotropic glutamate receptor 2; MGR3 HUMAN = human metabotropic glutamate receptor 3; MGR6 HUMAN = human metabotropic glutamate receptor 6; MGR4 HUMAN = human metabotropic glutamate receptor 4; MGR7 HUMAN = human metabotropic glutamate receptor 7; MGR8 HUMAN = human metabotropic glutamate receptor 8; MGR1 HUMAN = human metabotropic glutamate receptor 1; MGR5 HUMAN = human metabotropic glutamate receptor 5.

Figure 23 shows the coiled-coil domains in the C-termini of human GABABR1a and HG20. The upper sequence is from human



GABA<sub>B</sub>R1a and is positions 886-949 of SEQ.ID.NO.:21. The lower sequence is from HG20 and is positions 756-829 of SEQ.ID.NO.:2.

Figure 24 shows a comparison of the amino acid sequences of human GABA<sub>B</sub>R1a (referred to as "Human GABA-B1aR,"

5 SEQ.ID.NO.:21); the proteins encoded by two genes from *C. elegans* (*C. elegans* GABA-B1 = SEQ.ID.NO.:42 and *C. elegans* GABA-B2 = SEQ.ID.NO.:43); and HG20) (referred to as "Human GABA-B2," (SEQ.ID.NO.:2). The *C. elegans* genes have been predicted from *C.elegans* DNA sequence alone. ZK180 accession number: U58748 is  
10 predicted to be GABA-B2 and Y41G9. Contig99 and Y76F7.Contig73 were obtained from the Sanger *C. elegans* genomic sequence database and are predicted to be GABA-B1.

Figure 25A-D shows co-immunoprecipitation of the murine GABA<sub>B</sub>R1a and FLAG-HG20 receptor subunits and immunoblotting  
15 using reciprocal receptor subunit antibodies. Murine GABA<sub>B</sub>R1a and FLAG-HG20 receptors were expressed individually or co-expressed in COS-7 cells. Figure 25A shows the results of immunoblotting using an anti-murine GABA<sub>B</sub>R1a antibody. Immunoblot of the solubilized membranes using murine GABA<sub>B</sub>R1a antibodies 1713.1-1713.2 shows  
20 selective expression of murine GABA<sub>B</sub>R1a in murine GABA<sub>B</sub>R1a alone expressing cells (lane 3) and murine GABA<sub>B</sub>R1a /FLAG-HG20 co-expressing cells (lane 4), but not in mock transfected and FLAG-HG20 alone expressing cells (lanes 1 and 2). Staining of GABA<sub>B</sub>R1a subunits in co-expressing cells is more intense compared to cells expressing the  
25 GABA<sub>B</sub>R1a subunit alone, suggesting that HG20 subunits facilitate GABA<sub>B</sub>R1a expression. Figure 25B shows the results of immunoblotting using an anti-FLAG-HG20 antibody. Immunoblotting of the solubilized membranes using the anti-FLAG-HG20 antibody shows selective expression of FLAG-HG20 subunits in FLAG-HG20  
30 alone expressing cells (lane 6) and murine GABA<sub>B</sub>R1a /FLAG-HG20 co-expressing cells (lane 8), but not in mock transfected and murine GABA<sub>B</sub>R1a alone expressing cells (lanes 5 and 7). Staining of HG20 subunits in co-expressing cells is more intense compared to cells expressing the HG20 subunit alone, suggesting that GABA<sub>B</sub>R1a  
35 subunits facilitate HG20 expression. Figure 25C shows the results of immunoprecipitation with an anti-FLAG-HG20 antibody followed by

immunoblotting with an anti-murine GABA<sub>B</sub>R1a antibody. GABA<sub>B</sub>R1a /HG20 heterodimers are observed only in murine GABA<sub>B</sub>R1a /FLAG-HG20 co-expressing cells due to the fact that the GABA<sub>B</sub>R1a subunit was co-immunoprecipitated with the FLAG-HG20 subunit using the  
5 FLAG antibody and detected with GABA<sub>B</sub>R1a antibodies (lane 12). GABA<sub>B</sub>R1a subunits are not detected in mock-transfected cells and cells expressing GABA<sub>B</sub>R1a alone or FLAG-HG20 (lanes 9-11). Figure 25D shows the results of immunoprecipitation with an anti-murine GABA<sub>B</sub>R1a antibody followed by immunoblotting with an anti-FLAG-HG20 antibody. GABA<sub>B</sub>R1a /HG20 heterodimers are observed only in  
10 murine GABA<sub>B</sub>R1a /FLAG-HG20 co-expressing cells due to the fact that the FLAG-HG20 subunit was co-immunoprecipitated using the GABA<sub>B</sub>R1a antibodies and detected with FLAG antibody (lane 16). No FLAG-HG20 subunits are detected in mock-transfected cells or cells  
15 expressing murine GABA<sub>B</sub>R1a alone or FLAG-HG20 (lanes 13-15). The immunoblots shown are from 1-3 independent experiments.

Figure 26A-B shows some of the motifs in the N-termini of GABA<sub>B</sub> receptor subunits and related genes. Figure 26A shows an alignment of murine GABA<sub>B</sub>R1a (mGABA<sub>B</sub>1a; a portion of  
20 SEQ.ID.NO.:20), human GABA<sub>B</sub>R1a (hGABA<sub>B</sub>1a; a portion of SEQ.ID.NO.:21), HG20 (hGABA<sub>B</sub>2; a portion of SEQ.ID.NO.:2), metabotropic glutamate receptor 1 (mGluR1; SEQ.ID.NO.:44), and two *E. coli* proteins (LivK (SEQ.ID.NO.:45) and LivBP (SEQ.ID.NO.:46)). Figure 26B is a schematic drawing showing the location of the various  
25 motifs in murine GABA<sub>B</sub>R1a that are expected to be involved in heterodimer formation of GABA<sub>B</sub>R1a with HG20.

Figure 27 shows an expanded view of the coiled-coil region of homology between HG20 (hGABA<sub>B</sub>2; shown is a portion of  
SEQ.ID.NO.:2) and murine GABA<sub>B</sub>R1a (mGABA<sub>B</sub>1a; a portion of  
30 SEQ.ID.NO.:20). Also shown is the corresponding region of human GABA<sub>B</sub>R1a (hGABA<sub>B</sub>1a; a portion of SEQ.ID.NO.:21).

## DETAILED DESCRIPTION OF THE INVENTION

For the purposes of this invention:

35 "Substantially free from other proteins" means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%,

free of other proteins. Thus, for example, an HG20 protein preparation that is substantially free from other proteins will contain, as a percent of its total protein, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of non-HG20 proteins. Whether a given HG20 protein preparation is substantially free from other proteins can be determined by such conventional techniques of assessing protein purity as, *e.g.*, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) combined with appropriate detection methods, *e.g.*, silver staining or immunoblotting.

“Substantially free from other nucleic acids” means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other nucleic acids. Thus, for example, an HG20 DNA preparation that is substantially free from other nucleic acids will contain, as a percent of its total nucleic acid, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of non-HG20 nucleic acids. Whether a given HG20 DNA preparation is substantially free from other nucleic acids can be determined by such conventional techniques of assessing nucleic acid purity as, *e.g.*, agarose gel electrophoresis combined with appropriate staining methods, *e.g.*, ethidium bromide staining, or by sequencing.

An HG20 polypeptide has “substantially the same biological activity” as native HG20 (*i.e.*, SEQ.ID.NO.:2) if that polypeptide has a  $K_d$  for a ligand that is no more than 5-fold greater than the  $K_d$  of native HG20 for the same ligand. An HG20 polypeptide also has “substantially the same biological activity” as HG20 if that polypeptide can form heterodimers with either a GABABR1a or GABABR1b polypeptide, thus forming a functional GABAB receptor.

“Functional GABAB receptor” refers to a heterodimer of HG20 and either GABABR1a or GABABR1b where the heterodimer displays a functional response when exposed to GABA agonists. Examples of functional responses are: pigment aggregation in *Xenopus* melanophores, modulation of cAMP levels, coupling to inwardly rectifying potassium channels, mediation of late inhibitory postsynaptic potentials in neurons, increase in potassium conductance, and decrease in calcium conductance. One skilled in the art would be familiar with a

variety of methods of measuring the functional responses of G-protein coupled receptors such as the GABA<sub>B</sub> receptor (see, e.g., Lerner, 1994, Trends Neurosci. 17:142-146 [changes in pigment distribution in melanophore cells]; Yokomizo et al., 1997, Nature 387:620-624 [changes in cAMP or calcium concentration; chemotaxis]; Howard et al., 1996, Science 273:974-977 [changes in membrane currents in *Xenopus* oocytes]; McKee et al., 1997, Mol. Endocrinol. 11:415-423 [changes in calcium concentration measured using the aequorin assay]; Offermanns & Simon, 1995, J. Biol. Chem. 270:15175, 15180 [changes in inositol phosphate levels]). Depending upon the cells in which heterodimers of HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b are expressed, and thus the G-proteins with which the heterodimers are coupled, certain of such methods may be appropriate for measuring the functional responses of such heterodimers. It is well with the competence of one skilled in the art to select the appropriate method of measuring functional responses for a given experimental system.

A GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b polypeptide has "substantially the same biological activity" as a native GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b polypeptide if that polypeptide has a  $K_d$  for an amino acid, amino acid analogue, GABA<sub>B</sub> receptor agonist, or GABA<sub>B</sub> receptor antagonist such as CGP71872, GABA, saclofen, (-)baclofen, or (L)-glutamic acid that is no more than 5-fold greater than the  $K_d$  of a native GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b polypeptide for the same amino acid, amino acid analogue, GABA<sub>B</sub> receptor agonist, or GABA<sub>B</sub> receptor antagonist. A GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b polypeptide also has "substantially the same biological activity" as a native GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b polypeptide if that polypeptide can form heterodimers with an HG20 polypeptide, thus forming a functional GABA<sub>B</sub> receptor. Native GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b polypeptides include the murine GABA<sub>B</sub>R1a sequence shown as SEQ.ID.NO.:20; the rat GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b polypeptides disclosed in Kaupmann et al., 1997, Nature 386:239-246; the human GABA<sub>B</sub>R1a sequence disclosed in GenBank accession number AJ012185 (SEQ.ID.NO.:21); and the protein encoded by the DNA sequence disclosed in GenBank accession number Y11044 (SEQ.ID.NO.:23).

A "conservative amino acid substitution" refers to the replacement of one amino acid residue by another, chemically similar,

amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (e.g., arginine for lysine; glutamic acid  
5 for aspartic acid).

The present invention relates to the identification and cloning of HG20, a novel G-protein coupled receptor-like protein that represents a subunit for the GABA<sub>B</sub> receptor. The present invention provides DNA encoding HG20 that is substantially free from other  
10 nucleic acids. The present invention also provides recombinant DNA molecules encoding HG20 as well as isolated DNA molecules encoding HG20. Following the cloning of HG20 by the present inventors, a sequence highly similar to the sequence of HG20 was deposited in GenBank by Clark et al. (GenBank accession number AF056085), by  
15 White et al. (GenBank accession number AJ012188), and by Borowsky et al. (GenBank accession number AF074483). Two ESTs (GenBank accession number T07621, deposited June 30, 1993, and GenBank accession number Z43654, deposited September 21, 1995) each contain partial sequences of HG20 cDNA.

20 The present invention provides a DNA molecule substantially free from other nucleic acids comprising the nucleotide sequence shown in Figure 1 as SEQ.ID.NO.:1. Analysis of SEQ.ID.NO.:1 revealed that it contains a long open reading frame at positions 293-3,115. Thus, the present invention also provides a DNA  
25 molecule substantially free from other nucleic acids comprising the nucleotide sequence of positions 293-3,115 of SEQ.ID.NO.:1. The present invention also provides an isolated DNA molecule comprising the nucleotide sequence of positions 293-3,115 of SEQ.ID.NO.:1.

Sequence analysis of the open reading frame of the HG20  
30 DNA revealed that it encodes a protein of 941 amino acids with a calculated molecular weight of 104 kd and a predicted signal peptide. The predicted amino acid sequence of HG20 is 36% identical to the metabotropic GABA receptor-like sequence GABA<sub>B</sub>R1a described in Kaupmann (see above) throughout the entire sequence, and thus HG20  
35 most likely represents a novel metabotropic GABA receptor or receptor subunit. *In situ* hybridization showed that HG20 RNA is highly expressed in the cortex, thalamus, hippocampus, and cerebellum of the

brain, showing overlapping distribution with GABA<sub>B</sub>1a RNA as judged by *in situ* hybridization as well as with the expression of GABA<sub>B</sub> receptors as judged by pharmacological studies. HG20 RNA exhibits restricted distribution in the periphery, with low abundance of the 6.5 kb  
5 RNA in the heart, spleen, and pancreas and high levels in the adrenal gland. HG20 recombinantly expressed in COS-1 cells showed no specific binding for [<sup>3</sup>H](+)-baclofen, and when expressed in *Xenopus* oocyte and *Xenopus* melanophore functional assays, showed no activity to GABA, (-)-baclofen, and glutamic acid.

10 The novel DNA sequences of the present invention encoding HG20, in whole or in part, can be linked with other DNA sequences, *i.e.*, DNA sequences to which HG20 is not naturally linked, to form "recombinant DNA molecules" containing HG20. Such other sequences can include DNA sequences that control transcription or translation  
15 such as, *e.g.*, translation initiation sequences, promoters for RNA polymerase II, transcription or translation termination sequences, enhancer sequences, sequences that control replication in microorganisms, or that confer antibiotic resistance. The novel DNA sequences of the present invention can be inserted into vectors such as  
20 plasmids, cosmids, viral vectors, or yeast artificial chromosomes.

The present invention also includes isolated forms of DNA encoding HG20. By "isolated DNA encoding HG20" is meant DNA encoding HG20 that has been isolated from a natural source or produced by recombinant means. Use of the term "isolated" indicates that DNA  
25 encoding HG20 is not present in its normal cellular environment. Thus, an isolated DNA encoding HG20 may be in a cell-free solution or placed in a different cellular environment from that in which it occurs naturally. The term isolated does not imply that isolated DNA encoding HG20 is the only DNA present, but instead means that isolated DNA  
30 encoding HG20 is at least 95% free of non-nucleic acid material (*e.g.*, proteins, lipids, carbohydrates) naturally associated with the DNA encoding HG20. Thus, DNA encoding HG20 that is expressed in bacteria or even in eukaryotic cells which do not naturally (*i.e.*, without human intervention) contain it through recombinant means is "isolated  
35 DNA encoding HG20."

Included in the present invention are DNA sequences that hybridize to SEQ.ID.NO.:1 under stringent conditions. By way of example, and not limitation, a procedure using conditions of high stringency is as follows: Prehybridization of filters containing DNA is carried out for 2 hr. to overnight at 65°C in buffer composed of 6X SSC, 5X Denhardt's solution, and 100 µg/ml denatured salmon sperm DNA. Filters are hybridized for 12 to 48 hrs at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10<sup>6</sup> cpm of <sup>32</sup>P-labeled probe. Washing of filters is done at 37°C for 1 hr in a solution containing 2X SSC, 0.1% SDS. This is followed by a wash in 0.1X SSC, 0.1% SDS at 50°C for 45 min. before autoradiography.

Other procedures using conditions of high stringency would include either a hybridization carried out in 5XSSC, 5X Denhardt's solution, 50% formamide at 42°C for 12 to 48 hours or a washing step carried out in 0.2X SSPE, 0.2% SDS at 65°C for 30 to 60 minutes.

Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in, *e.g.*, Sambrook, Fritsch, and Maniatis, 1989, Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press. In addition to the foregoing, other conditions of high stringency which may be used are well known in the art.

Another aspect of the present invention includes host cells that have been engineered to contain and/or express DNA sequences encoding HG20. Such recombinant host cells can be cultured under suitable conditions to produce HG20. An expression vector containing DNA encoding HG20 can be used for expression of HG20 in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Cell lines derived from mammalian species which are suitable for recombinant expression of HG20 and which are commercially available, include but are not limited to, L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji

(ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, and *Xenopus* oocytes. In particular embodiments, the recombinant cells expressing HG20 protein co-express a GABABR1a or GABABR1b protein, thus forming a functional GABAB receptor comprising a heterodimer of HG20 and either GABABR1a or GABABR1b. In particular embodiments, the recombinant cells have been transfected with expression vectors that direct the expression of HG20 and GABABR1a or GABABR1b.

Cells that are particularly suitable for expression of the HG20 protein are melanophore pigment cells from *Xenopus laevis*. Such melanophore pigment cells can be used for functional assays that employ recombinant expression of HG20 in a manner similar to the use of such melanophore pigment cells for the functional assay of other recombinant GPCRs (Graminski et al., 1993, J. Biol. Chem. 268:5957-5964; Lerner, 1994, Trends Neurosci. 17:142-146; Potenza & Lerner, 1992, Pigment Cell Res. 5:372-378; Potenza et al., 1992, Anal. Biochem. 206:315-322). Especially preferred are *Xenopus* melanophore pigment cells co-expressing HG20 and GABABR1a or GABABR1b, in which HG20 has formed a heterodimer with GABABR1a or GABABR1b, thus forming a functional GABAB receptor. The presence of functional GABAB receptors in such cells can be determined by the use of assays such as the pigment aggregation assay described herein. Other assays that reflect a decrease in cAMP levels mediated by exposure to GABA or other agonists of GABAB receptors would also be suitable.

Also preferred are stably or transiently transfected HEK293 cells co-expressing HG20 and GABABR1a or GABABR1b, in which HG20 has formed a heterodimer with GABABR1a or GABABR1b, thus forming a functional GABAB receptor. The presence of functional GABAB receptors in such cells can be determined by the use of assays such as those that measure cAMP levels as described herein.

Also preferred are *Xenopus* oocytes co-expressing HG20 and GABABR1a or GABABR1b, in which HG20 has formed a heterodimer with GABABR1a or GABABR1b, thus forming a functional GABAB receptor. The presence of functional GABAB receptors in such



cells can be determined by the use of assays that measure coupling of functional GABA<sub>B</sub> receptors comprising heterodimers of HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b to inwardly rectifying potassium channels (especially the Kir3 family).

5 In order to produce the above-described cells co-expressing HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, expression vectors comprising DNA encoding HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b can be transfected into the cells. HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b can be transfected separately, each on its own expression vector, or, alternatively, a single  
10 expression vector encoding both HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b can be used.

A variety of mammalian expression vectors can be used to express recombinant HG20, GABA<sub>B</sub>R1a, or GABA<sub>B</sub>R1b in mammalian cells. Commercially available mammalian expression vectors which  
15 are suitable include, but are not limited to, pMC1neo (Stratagene), pSG5 (Stratagene), pcDNA1 and pcDNA1amp, pcDNA3, pcDNA3.1, pCR3.1 (Invitrogen), EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), and the PT7TS oocyte  
20 expression vector (or similar expression vectors containing the globin 5' UTR and the globin 3' UTR). The choice of vector will depend upon cell type used, level of expression desired, and the like. Following expression in recombinant cells, HG20, GABA<sub>B</sub>R1a, GABA<sub>B</sub>R1b, or heterodimers of HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b can be  
25 purified to a level that is substantially free from other proteins by conventional techniques, *e.g.*, salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography, hydrophobic interaction chromatography, and preparative gel electrophoresis. Also, membrane preparations  
30 comprising HG20, GABA<sub>B</sub>R1a, GABA<sub>B</sub>R1b, or heterodimers of HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b can be prepared. Especially preferred are membrane preparations that comprise heterodimers of HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in which the heterodimers represent functional GABA<sub>B</sub> receptors.

35 The present invention includes a method of producing HG20 protein comprising:

(a) transfecting a host cell with an expression vector comprising DNA that encodes an HG20 protein;

(b) growing the host cells under conditions such that HG20 protein is produced; and

5 (c) recovering HG20 protein from the host cells.

In particular embodiments, the method of recovering HG20 protein involves obtaining membrane preparations that contain HG20 protein from the host cells. In particular embodiments, such membrane preparations contain heterodimers of HG20 protein and GABABR1a or  
10 GABABR1b protein that form functional GABAB receptors.

The present invention includes a method of expressing a truncated HG20 protein comprising:

(a) transfecting a host cell with an expression vector comprising DNA that encodes an HG20 protein that has been truncated  
15 at the amino or carboxyl terminus;

(b) culturing the transfected cells of step (a) under conditions such that the truncated HG20 protein is expressed.

Truncated HG20 proteins are those HG20 proteins in which contiguous portions of the N terminus or C terminus have been  
20 removed. For example, positions 52-941 of SEQ.ID.NO.:2 represents a truncated HG20 protein. Truncated HG20 proteins may be fused in frame to non-HG20 amino acid sequences, as, *e.g.*, in the FLAG-HG20 construct described herein.

The present invention includes a method of producing  
25 functional GABAB receptors in cells comprising:

(a) transfecting cells with:

(1) an expression vector that directs the expression of HG20 in the cells; and

(2) an expression vector that directs the  
30 expression of GABABR1a or GABABR1b in the cells;

(b) culturing the cells under conditions such that heterodimers of HG20 and GABABR1a or GABABR1b are formed where the heterodimers constitute functional GABAB receptors.

In particular embodiments of the above methods, the cells  
35 are eukaryotic cells. In other embodiments, the cells are mammalian cells. In still other embodiments, the cells are COS cells, *e.g.*, COS-7

cells (ATCC CRL 1651) or COS-1 cells (ATCC CRL 1650); HEK293 cells (ATCC CRL 1573); or *Xenopus* melanophores.

In particular embodiments, the HG20 protein comprises the amino acid sequence shown in SEQ.ID.NO.:2. In particular  
5       embodiments, the HG20 protein is a truncated HG20 protein. In particular embodiments, the truncated HG20 protein comprises amino acids 52-941 of SEQ.ID.NO.:2. In particular embodiments, the truncated HG20 protein is a chimeric HG20 protein.

The present invention includes HG20 protein substantially  
10       free from other proteins. The amino acid sequence of the full-length HG20 protein is shown in Figure 2 as SEQ.ID.NO.:2. Thus, the present invention includes polypeptides comprising HG20 protein substantially free from other proteins where the polypeptides comprise the amino acid sequence SEQ.ID.NO.:2. The present invention also includes  
15       polypeptides comprising HG20 proteins lacking a signal sequence. Examples of amino acid sequences of HG20 proteins lacking a signal sequence are:

                  Positions 9-941 of SEQ.ID.NO.:2;  
                  Positions 35-941 of SEQ.ID.NO.:2;  
20               Positions 36-941 of SEQ.ID.NO.:2;  
                  Positions 38-941 of SEQ.ID.NO.:2;  
                  Positions 39-941 of SEQ.ID.NO.:2;  
                  Positions 42-941 of SEQ.ID.NO.:2;  
                  Positions 44-941 of SEQ.ID.NO.:2;  
25               Positions 46-941 of SEQ.ID.NO.:2;  
                  Positions 52-941 of SEQ.ID.NO.:2; and  
                  Positions 57-941 of SEQ.ID.NO.:2.

The present invention also includes DNA encoding the above-described HG20 proteins lacking a signal sequence. Thus, *e.g.*,  
30       the present invention includes a DNA molecule comprising a nucleotide sequence selected from the group consisting of:

                  Positions 293-3,115 of SEQ.ID.NO.:1;  
                  Positions 317-3,115 of SEQ.ID.NO.:1;  
                  Positions 395-3,115 of SEQ.ID.NO.:1;  
35               Positions 398-3,115 of SEQ.ID.NO.:1;  
                  Positions 404-3,115 of SEQ.ID.NO.:1;  
                  Positions 407-3,115 of SEQ.ID.NO.:1;

Positions 416-3,115 of SEQ.ID.NO.:1;  
Positions 422-3,115 of SEQ.ID.NO.:1;  
Positions 428-3,115 of SEQ.ID.NO.:1;  
Positions 446-3,115 of SEQ.ID.NO.:1; and  
Positions 461-3,115 of SEQ.ID.NO.:1.

5 As with many receptor proteins, it is possible to modify many of the amino acids of HG20, particularly those which are not found in the ligand binding domain, and still retain substantially the same biological activity as the original protein. Thus this invention includes modified HG20 polypeptides which have amino acid deletions, additions, or substitutions but that still retain substantially the same biological activity as native HG20. It is generally accepted that single amino acid substitutions do not usually alter the biological activity of a protein (see, *e.g.*, Molecular Biology of the Gene, Watson et al., 1987, 10 Fourth Ed., The Benjamin/Cummings Publishing Co., Inc., page 226; and Cunningham & Wells, 1989, Science 244:1081-1085). Accordingly, the present invention includes polypeptides where one amino acid substitution has been made in SEQ.ID.NO.:2 or in one of the HG20 polypeptides lacking a signal sequence listed above, wherein the polypeptides still retain substantially the same biological activity as native HG20. The present invention also includes polypeptides where two or more amino acid substitutions have been made in SEQ.ID.NO.:2 or in one of the HG20 polypeptides lacking a signal sequence listed above, wherein the polypeptides still retain substantially the same biological activity as native HG20. In particular, the present invention includes 20 embodiments where the above-described substitutions are conservative substitutions. In particular, the present invention includes embodiments where the above-described substitutions do not occur in the ligand-binding domain of HG20. In particular, the present invention includes 30 embodiments where amino acid changes have been made in the positions of HG20 where the amino acid sequence of HG20 differs from the amino acid sequence of GABABR1b (see Figure 8).

The present invention also includes C-terminal truncated forms of HG20, particularly those which encompass the extracellular portion of the receptor, but lack the intracellular signaling portion of the receptor. Such truncated receptors are useful in various binding assays described herein, for crystallization studies, and for structure-activity-

35

relationship studies. Accordingly, the present invention includes an HG20 protein substantially free from other proteins having the amino acid sequence of positions 1-480 of SEQ.ID.NO.:2.

O'Hara et al., 1993, Neuron 11:41-52 (O'Hara) reported that  
5 the amino terminal domains of several metabotropic glutamate receptors showed amino acid sequence similarities to the amino termini of several bacterial periplasmic binding proteins. O'Hara used this similarity to predict, and then experimentally confirm, that these amino terminal domains correspond to the location of the ligand binding  
10 domains of these metabotropic glutamate receptors.

The present inventors have discovered a region of amino acid sequence in the N-terminal domain of HG20 that is similar to the amino acid sequence of the bacterial periplasmic binding protein Leucine, Isoleucine, Valine (Alanine and Threonine) Binding Protein  
15 (LIVAT-BP) of *Pseudomonas aeruginosa*. See Figure 6. The region shown is about 25% identical between the two proteins. This is above the maximum identity of 17% reported by O'Hara between any one metabotropic glutamate receptor and any one periplasmic binding protein and indicates that the region of HG20 depicted is highly likely to  
20 contain the ligand binding domain.

Accordingly, the present invention includes a polypeptide representing the ligand binding domain of HG20 that includes amino acids 63-259 of SEQ.ID.NO.:2. Also provided are chimeric proteins comprising amino acids 63-259 of SEQ.ID.NO.:2.

25 Romano et al., 1996, J. Biol. Chem. 271:28612-28616 demonstrated that metabotropic glutamate receptors are often found as homodimers formed by an intermolecular disulfide bond. The location of the cysteines responsible for the disulfide bond was found to be in the amino terminal 17kD of the receptors. Transmembrane interactions  
30 may also contribute to functional GABA<sub>B</sub> receptor dimer formation, as previously reported for the dopamine D2 receptor and  $\beta$ 2-adrenergic receptor (Ng et al., 1996, Biochem. Biophys. Res. Comm. 227:200-204; Hebert et al., 1996, J. Biol. Chem. 271, 16384-16392). Accordingly, the present invention includes dimers of HG20 proteins. In particular  
35 embodiments, the HG20 protein has an amino acid selected from the group consisting of:

SEQ.ID.NO.:2;  
Positions 9-941 of SEQ.ID.NO.:2;  
Positions 35-941 of SEQ.ID.NO.:2;  
Positions 36-941 of SEQ.ID.NO.:2;  
5 Positions 38-941 of SEQ.ID.NO.:2;  
Positions 39-941 of SEQ.ID.NO.:2;  
Positions 42-941 of SEQ.ID.NO.:2;  
Positions 44-941 of SEQ.ID.NO.:2;  
Positions 46-941 of SEQ.ID.NO.:2;  
10 Positions 52-941 of SEQ.ID.NO.:2;  
Positions 57-941 of SEQ.ID.NO.:2; and  
Positions 1-480 of SEQ.ID.NO.:2.

It has been found that, in some cases, membrane spanning regions of receptor proteins can be used to inhibit receptor function (Ng  
15 et al., 1996, Biochem. Biophys. Res. Comm. 227:200-204; Hebert et al., 1996, J. Biol. Chem. 271, 16384-16392; Lofts et al., Oncogene 8:2813-2820). Accordingly, the present invention provides peptides derived from the seven membrane spanning regions of HG20 and their use to inhibit HG20 or GABAB receptor function. Such peptides can include the whole  
20 or parts of the membrane spanning domains.

The present invention also includes isolated forms of HG20 proteins. By "isolated HG20 protein" is meant HG20 protein that has been isolated from a natural source or produced by recombinant means. Use of the term "isolated" indicates that HG20 protein is not present in  
25 its normal cellular environment. Thus, an isolated HG20 protein may be in a cell-free solution or placed in a different cellular environment from that in which it occurs naturally. The term isolated does not imply that an isolated HG20 protein is the only protein present. but instead means that an isolated HG20 protein is at least 95% free of non-amino  
30 acid material (e.g., nucleic acids, lipids, carbohydrates) naturally associated with the HG20 protein. Thus, an HG20 protein that is expressed through recombinant means in bacteria or even in eukaryotic cells which do not naturally (i.e., without human intervention) express it is an "isolated HG20 protein."

35 The present invention also includes chimeric HG20 proteins. By chimeric HG20 protein is meant a contiguous polypeptide sequence of HG20 fused in frame to a polypeptide sequence of a non-

HG20 protein. For example, the N-terminal domain and seven transmembrane spanning domains of HG20 fused at the C-terminus in frame to a G protein would be a chimeric HG20 protein. Another example of a chimeric HG20 protein would be a polypeptide comprising the FLAG epitope fused in frame at the amino terminus of amino acids 52-941 of SEQ.ID.NO.:2.

The present invention also includes HG20 proteins that are in the form of multimeric structures, *e.g.*, dimers. Such multimers of other metabotropic G-protein coupled receptors are known (Hebert et al., 1996, J. Biol. Chem. 271, 16384-16392; Ng et al., 1996, Biochem. Biophys. Res. Comm. 227, 200-204; Romano et al., 1996, J. Biol. Chem. 271, 28612-28616).

Preferred forms of dimers of HG20 are heterodimers comprising HG20 and other G-protein coupled receptors (GPCRs). Such GPCRs could be, *e.g.*, other subunits of GABA<sub>B</sub> receptors, proteins from *C. elegans* showing homology to HG20 (see Figure 24), or human GPCRs that are homologs of the *C. elegans* proteins. Particularly preferred forms of heterodimers are heterodimers of HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b. It has been found by the present inventors that such heterodimers exhibit functional properties of GABA<sub>B</sub> receptors while monomers or homodimers of HG20, GABA<sub>B</sub>R1a, or GABA<sub>B</sub>R1b do not exhibit functional properties. Another likely heterodimer partner for HG20 is the protein corresponding to the sequence deposited in GenBank at accession number 3776096.

The strongest evidence that functional GABA<sub>B</sub> receptors require both HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b comes from studies demonstrating that co-transfection and co-expression of both HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b is necessary in order for the detection of GABA<sub>B</sub> receptor functional responses. Transfection and expression of HG20, GABA<sub>B</sub>R1a, or GABA<sub>B</sub>R1b alone does not lead to the production of functional GABA<sub>B</sub> receptors.

For example, in *Xenopus* melanophores co-expressing HG20 and GABA<sub>B</sub>R1a, but not in melanophores expressing HG20 or GABA<sub>B</sub>R1a alone, or in mock transfected melanophores, GABA mediated a dose-dependent pigment aggregation response that could be inhibited with the GABA<sub>B</sub> receptor specific CGP71872 antagonist. This pigment aggregation response is associated with a decrease in

intracellular cAMP levels. Such a decrease has been confirmed in HEK293 cells. Also, co-expression of HG20 and GABA<sub>B</sub>R1a in *Xenopus* oocytes resulted in the stimulation of inwardly rectifying potassium currents (Kirs). Native functional GABA<sub>B</sub> receptors have been reported  
5 to couple to Kirs (Misgeld et al., 1995, Prog. Neurobiol. 46:423-462).

Consistent with the need for both HG20 and GABA<sub>B</sub>R1a for detection of functional GABA<sub>B</sub> receptors in transfected cells, the present inventors have demonstrated that HG20 and GABA<sub>B</sub>R1a form  
10 heterodimers by immunoprecipitation of HG20 followed by immunoblotting with a GABA<sub>B</sub>R1a antibody.

That a functional GABA<sub>B</sub> receptor requires both HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b is also suggested by the observation that GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, recombinantly expressed in the absence of HG20, binds ligand with much reduced affinity compared to the  
15 affinity of native GABA<sub>B</sub> receptors. Also, characterization of the tissue distribution of each of the receptors by *in situ* hybridization histochemistry in rat brain revealed co-localization of HG20 and GABA<sub>B</sub>R1a transcripts in many brain regions, including cortex, at both the regional and cellular levels.

20 The *Xenopus* melanophore pigment aggregation/dispersion assay has been shown to be highly suitable for monitoring agonist activation of Gi-, Gq-, and Gs-coupled receptors (Potenza et al., 1992, Anal. Biochem. 206:315-322; Lerner, 1994, Trends Neurosci. 17:142-146). Agonist activation of Gi-coupled receptors expressed in melanophores results in  
25 pigment aggregation via a reduction in intracellular cAMP levels, whereas activation of Gs- and Gq-coupled receptors results in pigment dispersion via elevations in intracellular cAMP and calcium levels, respectively. Melanophores transfected separately with either GABA<sub>B</sub>R1a or HG20 showed no pigment aggregation or dispersion  
30 response following treatment with up to 1 mM concentrations of (L)-glutamic acid, GABA, or prototypic GABAergic agonists: (-)baclofen, 3-aminopropyl-(methyl)phosphonic acid, cis-4-aminocrotonic acid, piperidine-4-sulfonic acid (data not shown). Similarly, both receptors failed to couple to K<sup>+</sup> channels in *Xenopus* oocytes under patch-clamp  
35 conditions when transfected separately (data not shown). However, in melanophores transiently co-transfected with GABA<sub>B</sub>R1a and HG20, GABA mediated a dose-dependent aggregation response with an IC<sub>50</sub>



value of 3-7  $\mu$ M (n=3). This aggregation was absent in mock-transfected cells and in cells transfected with GABA $\beta$ R1a or HG20 alone (Figure 11). The GABA-mediated activity represented 42-56% (n=3) of a control cannabinoid receptor subtype 2 response (Figure 11, inset), and could be inhibited by the CGP71872 antagonist (n=3), indicating it was GABA $\beta$  receptor specific (Figure 11). GABA $\beta$ R1a was expressed by subcloning full-length GABA $\beta$ R1a into the NheI-NotI site of pcDNA3.1 or pCIneo; HG20 was expressed as a FLAG-HG20 chimeric protein. See Examples 11 and 20 for further experimental details of expression vectors used, transfection conditions, assay conditions, *etc.* for the above-described co-expression studies.

The functional data arising from co-expression of GABA $\beta$ R1a and HG20 receptors were confirmed in HEK293 cells. HEK293 cells transfected with and stably expressing GABA $\beta$ R1a and HG20 were selected based on expression of receptor message as determined by dot blot analyses. In cell lines stably expressing the individual receptors, we observed small and inconsistent responses in assays to examine agonist-mediated modulation of cAMP synthesis. However, transient transfection of HEK293 cells stably expressing GABA $\beta$ R1a (rgb1a-50) with an HG20 expression plasmid and transient transfection of HEK293 cells stably expressing HG20 (hgb2-42) with a GABA $\beta$ R1a expression plasmid significantly enhanced the ability of baclofen and GABA to inhibit forskolin-stimulated cAMP synthesis. Rgb1a-50 cells transfected with HG20 exhibited a 28% reduction in forskolin-stimulated cAMP synthesis with 30  $\mu$ M baclofen and a 40% decrease with 30  $\mu$ M GABA plus 100  $\mu$ M aminooxyacetic acid (AOAA; a GABA transaminase inhibitor) and 100  $\mu$ M nipecotic acid (a GABA uptake inhibitor) (Figure 12B). A 34% reduction in forskolin-stimulated cAMP synthesis was observed for hgb2-42 cells transfected with GABA $\beta$ R1a treated with baclofen and a 43% decrease was observed for GABA plus AOAA and nipecotic acid (Figure 12B). While inhibition of cAMP synthesis was sometimes observed with rgb1a-50 cells transfected with GABA $\beta$ R1a and hgb2-42 cells transfected with HG20, these effects were small and inconsistent (0-20% inhibition; Figure 12B). Neither baclofen nor GABA plus AOAA and nipecotic acid in the absence of forskolin had any affect on cAMP synthesis (Figure 12B). In addition,

wild-type HEK293 cells did not exhibit baclofen- or GABA-mediated inhibition of forskolin-stimulated cAMP synthesis (Figure 12B). These data demonstrate that the functional GABA<sub>B</sub> receptor requires both GABA<sub>B</sub>R1a and HG20. For experimental details of these studies in

5 HEK293 cells, see Example 12.

Co-expression of the GABA<sub>B</sub>R1a and HG20 with the inwardly rectifying potassium channels Kir 3.1/3.2 in *Xenopus* oocytes resulted in a significant stimulation of inwardly rectifying potassium current (Kir) in response to GABA [301 +/- 20.6 %, (n=3) increase over  
10 control current] measured at -80 mV which could subsequently be washed out with control solution (Figure 13). Modulation of Kir 3.1/3.2 was not seen in oocytes expressing GABA<sub>B</sub>R1a or HG20 individually, even in the presence of Gi $\alpha$ 1 (Figure 13). See Example 21 for details.

To determine whether receptor intermolecular interactions  
15 accounted for the functional activity that was observed following the co-expression of recombinant GABA<sub>B</sub>R1a and HG20, membranes from cells co-expressing GABA<sub>B</sub>R1a and HG20 or the individual proteins were first immunoprecipitated using anti-FLAG antibodies (to detect the recombinant FLAG-HG20 chimeric proteins) followed by  
20 immunoblotting with a GABA<sub>B</sub>R1a-specific antibody. As seen in Figure 14, lanes 1-3, no GABA<sub>B</sub>R1a immunoreactivity was detected in samples prepared from mock vector transfected cells, FLAG-HG20 alone expressing cells, and GABA<sub>B</sub>R1a alone expressing cells.  
immunoprecipitated with the FLAG-antibody. Since immunoreactive  
25 species were detected only in cells co-expressing HG20 and GABA<sub>B</sub>R1a, this experiment demonstrates that HG20 and GABA<sub>B</sub>R1a can only be co-immunoprecipitated as part of a complex (Figure 14, lane 4). Based on the predicted molecular mass of a heterodimer of HG20 and GABA<sub>B</sub>R1a, the ~250+ and ~130 kDa species may represent a  
30 heterodimer and GABA<sub>B</sub>R1a monomers, respectively. The stability of the HG20/GABA<sub>B</sub>R1a heterodimer in denaturing and reducing conditions suggests that SDS-stable transmembrane interactions form the heterodimer, as reported previously for  $\beta$ 2 adrenergic and dopamine D2 receptors (Ng et al., 1996, Biochem. Biophys. Res. Comm. 227:200-204; Hebert et al., 1996, J. Biol. Chem. 271, 16384-16392). The monomer might  
35 result from partial disruption, subsequent to immunoprecipitation, of N-terminal Sushi repeats, C-terminal alpha-helical interacting domains

(e.g., coiled-coils) present in HG20 and GABA<sub>B</sub>R1a subunits, transmembrane interactions, or disulfide bonds that contribute to forming the heterodimer.

Particular examples of such regions likely to be involved in forming the heterodimer are shown in Figure 23. Regions such as those shown in Figure 23, as well as polypeptides comprising such regions are expected to be useful for the purpose of modulating the formation of heterodimers involving HG20 and thus controlling GABA<sub>B</sub> receptor activity. Accordingly, the present invention includes polypeptides comprising the coiled-coil domains of HG20, GABA<sub>B</sub>R1a, and GABA<sub>B</sub>R1b. In particular, the present invention includes polypeptides comprising an amino acid sequence selected from the group consisting of: positions 756-829 of SEQ.ID.NO.:2; positions 779-814 of SEQ.ID.NO.:2; positions 886-949 of SEQ.ID.NO.:21; and positions 889-934 of SEQ.ID.NO.:21; where the polypeptides do not contain other contiguous amino acid sequences longer than 5 amino acids from a GABA<sub>B</sub> receptor subunit. The present invention also includes heterodimers of such polypeptides. In more general terms, the present invention includes comprising a coiled-coil domain from a first GABA<sub>B</sub> receptor subunit and no other contiguous amino acid sequences longer than 5 amino acids from the first GABA<sub>B</sub> receptor subunit where the coiled-coil domain is present in the C-terminus of the first GABA<sub>B</sub> receptor subunit and mediates heterodimerization of the first GABA<sub>B</sub> receptor subunit with a second GABA<sub>B</sub> receptor subunit.

In addition to the coiled-coil domains discussed above, a variety of regions of HG20 and GABA<sub>B</sub>R1a are expected to be important for heterodimer formation. Motif analysis of the N-terminus of murine GABA<sub>B</sub>R1a revealed seven consensus N-linked glycosylation sites and three putative short consensus repeats (SCRs) of ~60 amino acids each: amino acids 27-96 and amino acids 102-157 (GABA<sub>B</sub>R1a specific), and amino acids 183-245 (common to GABA<sub>B</sub>R1b (Kaupmann et al., 1997, Nature 386:239-246) and HG20 (Jones et al., 1998, Nature 396:674-679; White et al., 1998, Nature 396:679-682; Kaupmann et al., 1998, Nature 396:683-687; Kuner et al., 1999, Science 283:74-77) not described previously (Figure 26A-B). Since SCRs are known to play important roles in protein-protein interactions in a wide variety of complement

proteins, adhesion proteins, and selectins (Chou and Henrikson, 1997, J. Protein Chem. 16:765-773; Perkins et al., 1998, Biochemistry 27:4004-4012), of which the latter shows weak amino acid identity to murine GABABR1a, these SCRs, together with the coiled-coil domains  
5 discussed above in the carboxyl tails of GABABR1a and HG20 (Figure 23), are expected to be involved in the heterodimerization of GABABR1a and HG20.

Therefore, the present invention includes a polypeptide comprising an SCR domain from a first GABAB receptor subunit and  
10 no other contiguous amino acid sequences longer than 5 amino acids from the first GABAB receptor subunit where the SCR domain is present in the N-terminus of the first GABAB receptor subunit and mediates heterodimerization of the first GABAB receptor subunit with a second GABAB receptor subunit. In particular embodiments, the SCR  
15 is selected from the group consisting of: positions 27-96 of SEQ.ID.NO.:20; positions 102-157 of SEQ.ID.NO.:20; positions 183-245 of SEQ.ID.NO.:20; positions 28-97 of SEQ.ID.NO.:21; positions 103-158 of SEQ.ID.NO.:21; positions 184-246 of SEQ.ID.NO.:21; positions 4-22 of SEQ.ID.NO.:2; positions 23-49 of SEQ.ID.NO.:2; and positions 72-135 of  
20 SEQ.ID.NO.:2.

As in the metabotropic glutamate receptors (mGLURs), the second intracellular loop of murine GABABR1a is rich in basic amino acids which may play a role in G-protein-interactions (reviewed by Pin and Duvoisin, 1995, Neuropharmacology 34:1-26), and, as in the  
25 mGLURs, the carboxyl tail of murine GABABR1a contains a PDZ protein-interacting module (serine-arginine-valine, amino acids 953-955) which has been shown for mGLURs to play an important role in the interactions among the signaling components of synaptic junctions (Brakeman et al.1997, Nature 386:284-288). The murine GABABR1a  
30 receptor also contains potential protein kinase C and casein kinase II recognition sites predicted using ProSearch (Kolakowski et al., 1992, Biotechniques 13:919-921).

The present invention also relates to the identification and cloning of the murine GABABR1a receptor, the murine ortholog of the  
35 rat GABABR1a receptor described in Kaupmann et al., 1997, Nature 386:239-246 (Kaupmann). The present invention provides DNA encoding murine GABABR1a that is substantially free from other nucleic acids.

The present invention also provides recombinant DNA molecules encoding murine GABA<sub>B</sub>R1a.

The present invention provides a DNA molecule encoding murine GABA<sub>B</sub>R1a that is substantially free from other nucleic acids and comprises the nucleotide sequence shown in Figure 15 as SEQ.ID.NO.:19. The open reading frame of SEQ.ID.NO.:19, encoding mouse GABA<sub>B</sub>R1a protein, is positions 1-2,880, with positions 2,881-2,883 representing a stop codon. Thus, the present invention also provides a DNA molecule substantially free from other nucleic acids comprising the nucleotide sequence of positions 1-2,880 of SEQ.ID.NO.:19.

Sequence analysis of the open reading frame of the murine GABA<sub>B</sub>R1a DNA revealed that it encodes a mature protein (*i.e.*, lacking a signal sequence) of 942 amino acids with a predicted molecular weight of 106.5 kDa that is 99% identical to rat GABA<sub>B</sub>R1a (described in Kaupmann), with only six amino acid changes overall. Murine GABA<sub>B</sub>R1a protein shares 31% overall amino acid identity to HG20.

CGP71872 is a photoaffinity ligand specific for GABA<sub>B</sub>R1a receptors ( $K_d = 1.0 \pm 0.2$  nM) that can be cross-linked to rat GABA<sub>B</sub>R1a (Kaupmann et al., 1997, Nature 386:239-246). In crude membranes prepared from COS-7 cells transiently transfected with murine GABA<sub>B</sub>R1a, [<sup>125</sup>I]CGP71872 photolabelled a major band at ~130 kDa representing the mature (presumably glycosylated) protein and an additional band at approximately twice that molecular weight, possibly representing dimers (Figure 9). Ligand-binding species could also be detected with affinity purified GABA<sub>B</sub>R1a antibodies 1713.1 (raised against the peptide acetyl-DVNSRRDILPDYELKLC-amide; a portion of SEQ.ID.NO.:20) and 1713.2 (raised against the peptide acetyl-CATLHNPTRVKLF EK-amide; a portion of SEQ.ID.NO.:20) (Figure 9). In contrast, FLAG-tagged HG20 protein did not bind the high-affinity CGP71872 ligand, although expression of the protein was confirmed by immunoblot analysis (Figure 9).

Displacement of [<sup>125</sup>I]CGP71872 binding to recombinant murine GABA<sub>B</sub>R1a was in the appropriate rank order of potency for GABAergic ligands: CGP71872 > SKF-97541 (3-aminopropyl(methyl)-phosphinic acid) > GABA > (-)baclofen > saclofen > (L)-glutamic acid.. Interestingly, recombinant rat GABA<sub>B</sub>R1a exhibits 10-25 fold lower affinity for agonists than native GABA<sub>B</sub> receptors in brain (Kaupmann

et al., 1997, *Nature* 386:239). Although the reason for this discrepancy remains unclear, a recent report indicated that recombinant GABA<sub>B</sub>R1a may require additional cellular components for functional targeting to the plasma membrane (Couve et al., 1998, *J. Biol. Chem.* 273:26361-26367). Thus, GABA<sub>B</sub>R1a alone, without such additional components, might be expected to exhibit somewhat altered ligand binding characteristics.

In the binding experiments discussed above using GABA<sub>B</sub>R1a alone, surprisingly, dose-dependent displacement was not detected for (+)baclofen, and the affinities of agonists (GABA, SKF-97541, and (-)baclofen) and partial agonists ((+)baclofen, saclofen, (L)-glutamic acid) but not the affinity of antagonist (CGP71872) for the recombinant GABA<sub>B</sub>R1a were markedly lower compared to native receptors in rat brain (Table 1). Agonist affinities of co-expressed HG20 and GABA<sub>B</sub>R1a were examined in membranes prepared from cells co-expressing GABA<sub>B</sub>R1a and FLAG-tagged HG20. Competition of [<sup>125</sup>I]CGP71872 binding in these membranes showed recovery of high-affinity ligand binding comparable to native receptors in rat brain (Table 1). The simplest explanation for these results is that the high-affinity agonist binding pocket may comprise interactions between the N-terminal domains of HG20 and GABA<sub>B</sub>R1a that form the heterodimer.

Table 1

Ligand	rat cortex*	gb1a	gb1a/gb2
CGP71872	0.5 nM	0.52 - 0.67 nM	0.15 - 0.27 nM
GABA	2.5 uM	42.55 - 68.38 uM	1.77 - 2.55 uM
SKF-97541**	not determined	11.09 - 11.47 uM	0.80 - 0.96 uM
(-)Baclofen	0.5 uM	31.46 - 53.70 uM	3.92 - 7.78 uM
(+)Baclofen	not determined	no fit	1.25 - 3.94 mM
Saclofen	156 uM	280.5 - 365.0 uM	119.4 - 131.4 uM
L-Glutamate	not determined	119.4 - 285.0 mM	116.2 - 201.6 mM

\* reported by Kaupmann et al., (1997) *Nature* 386, 239-246  
 \*\* 3-aminopropyl(methyl)phosphinic acid

In Table 1, gb1a refers to GABA<sub>B</sub>R1a and gb1a/gb2 refers to HG20/ GABA<sub>B</sub>R1a heterodimers.

Co-localization studies were performed to determine if mRNAs for GABA<sub>B</sub>R1a and HG20 co-exist in the same cells in the brain. Figure 10A-B shows equivalent levels of GABA<sub>B</sub>R1a and HG20

hybridization in adjacent coronal sections of rat parietal cortex, indicating that messages for both receptors are expressed in this brain region. Radiolabelled and fluorescent probes for the two receptors were used to look at the cellular level where it was observed that message for both receptors is expressed in the same cells (Example 13 and Figure 10C-E). In the parietal cortex and all other major brain regions studied, including the hippocampus, thalamus, cerebellum, and vestibular ganglion, GABABR1a and HG20 mRNAs are co-localized in the same cells. These results suggest that the functional native GABAB receptors found in these cells involve both GABABR1a and HG20. Co-immunoprecipitation, functional, and anatomical data described herein converge to strongly support the argument that the native, functional GABAB receptor is a heterodimer of GABABR1a and HG20. This work is particularly exciting because it represents the first example of a heteromeric G protein-coupled receptor.

The novel murine GABABR1a DNA sequences of the present, in whole or in part, can be linked with other DNA sequences, *i.e.*, DNA sequences to which GABABR1a DNA is not naturally linked, to form "recombinant DNA molecules" encoding murine GABABR1a. Such other sequences can include DNA sequences that control transcription or translation such as, *e.g.*, translation initiation sequences, promoters for RNA polymerase II, transcription or translation termination sequences, enhancer sequences, sequences that control replication in microorganisms, or that confer antibiotic resistance. The novel DNA sequences of the present invention can be inserted into vectors such as plasmids, cosmids, viral vectors, or yeast artificial chromosomes.

The present invention also includes isolated forms of DNA encoding GABABR1a. By "isolated DNA encoding GABABR1a" is meant DNA encoding GABABR1a that has been isolated from a natural source or produced by recombinant means. Use of the term "isolated" indicates that DNA encoding GABABR1a is not present in its normal cellular environment. Thus, an isolated DNA encoding GABABR1a may be in a cell-free solution or placed in a different cellular environment from that in which it occurs naturally. The term isolated does not imply that isolated DNA encoding GABABR1a is the only DNA present, but instead means that isolated DNA encoding GABABR1a is at

least 95% free of non-nucleic acid material (*e.g.*, proteins, lipids, carbohydrates) naturally associated with the DNA encoding GABABR1a. Thus, DNA encoding GABABR1a that is expressed in bacteria or even in eukaryotic cells which do not naturally (*i.e.*, without  
5 human intervention) contain it through recombinant means is "isolated DNA encoding GABABR1a."

Another aspect of the present invention includes host cells that have been engineered to contain and/or express DNA sequences encoding murine GABABR1a. Such recombinant host cells can be  
10 cultured under suitable conditions to produce murine GABABR1a protein. An expression vector containing DNA encoding the murine GABABR1a protein can be used for expression of the murine GABABR1a protein in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria  
15 such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Cell lines derived from mammalian species which are suitable for recombinant expression of the murine  
20 GABABR1a protein and which are commercially available, include but are not limited to, L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa  
25 (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus melanophores*, and *Xenopus oocytes*.

A variety of mammalian expression vectors can be used to express recombinant murine GABABR1a in mammalian cells. Commercially available mammalian expression vectors which are  
30 suitable include, but are not limited to, pMC1neo (Stratagene), pSG5 (Stratagene), pcDNAI and pcDNAIamp, pcDNA3, pcDNA3.1, pCR3.1 (Invitrogen), EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pDBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), and the PT7TS oocyte  
35 expression vector (or similar expression vectors containing the globin 5' UTR and the globin 3' UTR). Following expression in recombinant cells,



the murine GABA<sub>B</sub>R1a protein can be purified by conventional techniques to a level that is substantially free from other proteins.

Other cells that are particularly suitable for expression of the murine GABA<sub>B</sub>R1a protein are immortalized melanophore pigment cells from *Xenopus laevis*. Such melanophore pigment cells can be used for functional assays using recombinant expression of murine GABA<sub>B</sub>R1a in a manner similar to the use of such melanophore pigment cells for the functional assay of other recombinant GPCRs (Graminski et al., 1993, J. Biol. Chem. 268:5957-5964; Lerner, 1994, Trends Neurosci. 17:142-146; Potenza & Lerner, 1992, Pigment Cell Res. 5:372-378; Potenza et al., 1992, Anal. Biochem. 206:315-322).

The present invention includes a method of producing the murine GABA<sub>B</sub>R1a protein comprising:

- (a) transfecting a host cell with a expression vector comprising DNA that encodes the murine GABA<sub>B</sub>R1a protein;
- (b) growing the host cells under conditions such that the murine GABA<sub>B</sub>R1a protein is produced; and
- (c) recovering the murine GABA<sub>B</sub>R1a protein from the host cells.

In particular embodiments, the method of recovering the murine GABA<sub>B</sub>R1a protein may involve obtaining membrane preparations from the host cells that contain the murine GABA<sub>B</sub>R1a protein. Such membrane preparations may contain heterodimers of GABA<sub>B</sub>R1a protein and HG20 protein that form functional GABA<sub>B</sub> receptors.

In particular embodiments, the cells are eukaryotic cells. In other embodiments, the cells are mammalian cells. In still other embodiments, the cells are COS cells, in particular COS-7 cells (ATCC CRL 1651), COS-1 cells (ATCC CRL 1650), HEK293 cells (ATCC CRL 1573), or *Xenopus* melanophores.

The present inventors have discovered that, when either HG20 or GABA<sub>B</sub>R1a subunits are recombinantly expressed separately, *i.e.*, in different cells, very little or no expression is observed. It is only when HG20 and GABA<sub>B</sub>R1a subunits are recombinantly co-expressed, *i.e.*, expressed in the same cells at the same time, that high level expression of HG20 and GABA<sub>B</sub>R1a is observed (see Figure 25). Given

the close relationship among GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20 (see Figure 24), and the close relationship that is expected to be found between other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b, it is believed that co-expression of HG20 and either GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b will also result in increased expression of HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b as compared to expression of these proteins separately.

Accordingly, the present invention includes a method of co-expressing HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b so as to result in an increase in expression of HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b as compared to expression when HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b are expressed separately. In particular embodiments, the level of expression of HG20, GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b is measured in the co-expressing cells. In particular embodiments, the level of expression of HG20, GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b is measured by immunoblot or by immunoprecipitation/immunoblotting methods.

Thus, the present invention includes a method of increasing expression of HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b comprising:

- (a) recombinantly expressing HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b in the same cells;
- (b) measuring the expression of HG20, GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b, where a measurement of

detectable expression of HG20, GABA<sub>B</sub>R1a, GABA<sub>B</sub>R1b, *C. elegans* genes related to GABA<sub>B</sub>R1a and HG20, or other isoforms of GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b indicates that increased expression has been achieved.

5 In particular embodiments, the measurement of expression is carried out by immunoblotting with or without immunoprecipitation.

In other embodiments, the method also comprises the steps of recombinantly expressing HG20 and GABA<sub>B</sub>R1a, GABA<sub>B</sub>R1b, *C. elegans* genes related to GABA<sub>B</sub>R1a and HG20, or other isoforms of  
10 GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b separately, measuring the level of expression of HG20, GABA<sub>B</sub>R1a, GABA<sub>B</sub>R1b, *C. elegans* genes related to GABA<sub>B</sub>R1a and HG20, or other isoforms of GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b in the separately expressing cells, and comparing the amount of expression of HG20, GABA<sub>B</sub>R1a, GABA<sub>B</sub>R1b, *C. elegans*  
15 genes related to GABA<sub>B</sub>R1a and HG20, or other isoforms of GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b in the separately expressing cells to the amount of expression of HG20, GABA<sub>B</sub>R1a, GABA<sub>B</sub>R1b, *C. elegans* genes related to GABA<sub>B</sub>R1a and HG20, or other isoforms of GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b in the co-expressing cells.

20 Accordingly, the present invention includes a a method of increasing expression of HG20 and GABA<sub>B</sub>R1a, GABA<sub>B</sub>R1b, *C. elegans* genes related to GABA<sub>B</sub>R1a and HG20, or other isoforms of GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b comprising:

(a) recombinantly expressing HG20 and GABA<sub>B</sub>R1a,  
25 GABA<sub>B</sub>R1b, *C. elegans* genes related to GABA<sub>B</sub>R1a and HG20, or other isoforms of GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b in the same cells to form co-expressing cells;

(b) recombinantly expressing HG20 and GABA<sub>B</sub>R1a, GABA<sub>B</sub>R1b, *C. elegans* genes related to GABA<sub>B</sub>R1a and HG20, or other  
30 isoforms of GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b in different cells to form separately expressing cells;

(c) measuring the expression of HG20, GABA<sub>B</sub>R1a, GABA<sub>B</sub>R1b, *C. elegans* genes related to GABA<sub>B</sub>R1a and HG20, or other isoforms of GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b in the co-expressing cells;

35 (d) measuring the expression of HG20, GABA<sub>B</sub>R1a, GABA<sub>B</sub>R1b, *C. elegans* genes related to GABA<sub>B</sub>R1a and HG20, or other

isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b in the separately expressing cells;

where if the amount of expression of HG20, GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other  
5 isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b is greater in the co-expressing cells as compared to the separately expressing cells, this indicates that increased expression has been achieved.

In particular embodiments, the measurement of expression is carried out by immunoblotting with or without  
10 immunoprecipitation.

The present invention includes murine GABA $\beta$ R1a protein substantially free from other proteins. The amino acid sequence of the full-length murine GABA $\beta$ R1a protein is shown in Figure 16 as SEQ.ID.NO.:20. Thus, the present invention includes polypeptides  
15 comprising the murine GABA $\beta$ R1a protein substantially free from other proteins having the amino acid sequence SEQ.ID.NO.:20. The present invention also includes murine GABA $\beta$ R1a protein lacking a signal sequence as well as DNA encoding such a protein. Such a murine GABA $\beta$ R1a protein lacking a signal sequence is represented by  
20 amino acids 18-960 of SEQ.ID.NO.:20.

The present invention includes modified murine GABA $\beta$ R1a polypeptides which have amino acid deletions, additions, or substitutions but that still retain substantially the same biological activity as native murine GABA $\beta$ R1a protein. The present invention  
25 includes polypeptides where one amino acid substitution has been made in SEQ.ID.NO.:20 or in a polypeptide represented by SEQ.ID.NO.:20 lacking a signal sequence, wherein the polypeptides still retain substantially the same biological activity as native murine GABA $\beta$ R1a protein. The present invention also includes polypeptides where two or  
30 more amino acid substitutions have been made in SEQ.ID.NO.:20 or in a polypeptide represented by SEQ.ID.NO.:20 lacking a signal sequence, wherein the polypeptides still retain substantially the same biological activity as native murine GABA $\beta$ R1a protein. In particular, the present invention includes embodiments where the above-described  
35 substitutions are conservative substitutions. In particular, the present invention includes embodiments where the above-described

substitutions do not occur in the ligand-binding domain of native murine GABA<sub>B</sub>R1a protein. In particular, the present invention includes embodiments where amino acid changes have been made in positions of native murine GABA<sub>B</sub>R1a protein where the amino acid sequence of native murine GABA<sub>B</sub>R1a protein differs from the amino acid sequence of HG20 when the amino acid sequences of native murine GABA<sub>B</sub>R1a protein and HG20 are aligned in a manner similar to the alignment of the amino acid sequences of GABA<sub>B</sub>R1b protein and HG20 shown in Figure 8.

The present invention also includes isolated forms of murine GABA<sub>B</sub>R1a proteins. By "isolated murine GABA<sub>B</sub>R1a protein" is meant murine GABA<sub>B</sub>R1a protein that has been isolated from a natural source or produced by recombinant means. Use of the term "isolated" indicates that murine GABA<sub>B</sub>R1a protein is not present in its normal cellular environment. Thus, an isolated murine GABA<sub>B</sub>R1a protein may be in a cell-free solution or placed in a different cellular environment from that in which it occurs naturally. The term isolated does not imply that an isolated murine GABA<sub>B</sub>R1a protein is the only protein present, but instead means that an isolated murine GABA<sub>B</sub>R1a protein is at least 95% free of non-amino acid material (e.g., nucleic acids, lipids, carbohydrates) naturally associated with the murine GABA<sub>B</sub>R1a protein. Thus, an murine GABA<sub>B</sub>R1a protein that is expressed in bacteria or even in eukaryotic cells which do not naturally (i.e., without human intervention) express it through recombinant means is an "isolated murine GABA<sub>B</sub>R1a protein."

The present invention also provides ligand-binding domains of murine GABA<sub>B</sub>R1a protein. A FASTA search of the database GenBank (bacterial division) using the N-terminal domain of murine GABA<sub>B</sub>R1a (amino acid positions 147-551 of SEQ.ID.NO.:20) as the probe reveals a match with the *E.coli* leucine-specific binding protein (livK) (22% identity over 339 amino acids), whereas no match to any bacterial amino acid binding protein is found using the receptor sequence inclusive of the region that includes the seven transmembrane domains (TM 1-7; amino acid positions 552-960) as a probe. The ligand-binding domain(s) of GABA<sub>B</sub>R1a was also experimentally determined. Photoaffinity [<sup>125</sup>I]CGP71872 labeling experiments provided direct physical evidence that the N-terminal extracellular domain but not a C-

terminal fragment of GABA<sub>B</sub>R1a (comprising TM1-7 and inclusive to the carboxyl tail) is responsible for ligand-binding (see Examples 14-19 and Figure 17B).

Accordingly, the present invention includes a polypeptide comprising the ligand binding domain of murine GABA<sub>B</sub>R1a. In preferred embodiments, the polypeptide comprises amino acids 147-551 of SEQ.ID.NO.:20.

The present invention includes methods of identifying compounds that specifically bind to the GABA<sub>B</sub> receptor, as well as compounds identified by such methods. The specificity of binding of compounds showing affinity for the GABA<sub>B</sub> receptor is shown by measuring the affinity of the compounds for recombinant cells expressing HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, or for membranes from such cells. Expression of the GABA<sub>B</sub> receptor and screening for compounds that bind to the GABA<sub>B</sub> receptor or that inhibit the binding of a known, radiolabeled ligand of the GABA<sub>B</sub> receptor, *e.g.*, an amino acid or a GABA analogue such as (-)baclofen, to these cells, or membranes prepared from these cells, provides an effective method for the rapid selection of compounds with high affinity for the GABA<sub>B</sub> receptor. Other radiolabeled ligands that might be used are ibotenic acid, the amino acids glutamate and glycine, other amino acids, decarboxylated amino acids, or any of the other GABA<sub>B</sub> receptor ligands disclosed herein or known in the art. Such ligands need not necessarily be radiolabeled but can also be nonisotopic compounds that can be used to displace bound radiolabeled compounds or that can be used as activators in functional assays. Compounds identified by the methods disclosed herein are likely to be agonists or antagonists of the GABA<sub>B</sub> receptor and may be peptides, proteins, or non-proteinaceous organic molecules.

Therefore, the present invention includes assays by which GABA<sub>B</sub> receptor agonists and antagonists can be identified. Methods for identifying agonists and antagonists of other receptors are well known in the art and can often be adapted to identify agonists and antagonists of the GABA<sub>B</sub> receptor. Accordingly, the present invention includes a method for determining whether a substance binds GABA<sub>B</sub> receptors

and is thus a potential agonist or antagonist of the GABA<sub>B</sub> receptor that comprises:

- (a) providing cells comprising an expression vector encoding HG20 and an expression vector encoding GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b;
  - (b) culturing the cells under conditions such that HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b are expressed and heterodimers of HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b are formed;
  - (c) exposing the cells to a labeled ligand of GABA<sub>B</sub> receptors in the presence and in the absence of the substance;
  - (d) measuring the binding of the labeled ligand to the heterodimers of HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the presence and in the absence of the substance;
- where if the amount of binding of the labeled ligand is less in the presence of the substance than in the absence of the substance, then the substance is a potential agonist or antagonist of GABA<sub>B</sub> receptors.

Examples of ligands of GABA<sub>B</sub> receptors are: CGP71872, GABA, saclofen, (-)baclofen, glycine, and (L)-glutamic acid.

- The present invention also includes a method for determining whether a substance is capable of binding to GABA<sub>B</sub> receptors, *i.e.*, whether the substance is a potential agonist or an antagonist of GABA<sub>B</sub> receptors, where the method comprises:
- (a) providing test cells comprising an expression vector encoding HG20 and an expression vector encoding GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b;
  - (b) culturing the test cells under conditions such that HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b are expressed and heterodimers of HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b are formed;
  - (c) exposing the test cells to the substance;
  - (d) measuring the amount of binding of the substance to the test cells;
  - (e) measuring the amount of binding of the substance to control cells;
  - (f) comparing the amount of binding of the substance to the test cells with the amount of binding of the substance to control cells;

where if the amount of binding of the substance to the test cells is greater than the amount of binding of the substance to control cells, then the substance is capable of binding to GABA<sub>B</sub> receptors;

5 where the control cells are essentially the same as the test cells except that the control cells do not comprise an expression vector encoding HG20 and an expression vector encoding GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b.

Once a substance has been identified by the above-described methods, determining whether the substance is an agonist or  
10 antagonist can then be accomplished by the use of functional assays such as those described herein.

In particular embodiments, the cells are transfected with an expression vector encoding HG20 and an expression vector encoding GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b.

15 In particular embodiments, the binding affinity of the substance for the test cells is determined. In particular embodiments, such binding affinity is between 1nM and 200 mM; preferably between 5 nM and 1 mM; more preferably between 10 nM and 100 μM; and even more preferably between 10 nM and 100 nM.

20 The conditions under which step (c) of the above-described methods is practiced are conditions that are typically used in the art for the study of protein-ligand interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

25 In a particular embodiment of the above-described methods, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), or *Xenopus* melanophores.

35 The assays described above can be carried out with cells that have been transiently or stably transfected with an expression vector encoding HG20 and an expression vector encoding GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b. Transfection is meant to include any method known in the



art for introducing HG20 and GABA $\beta$ R1a or GABA $\beta$ R1b into the test cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct, and electroporation. In particular embodiments, a single  
5 expression vector encodes HG20 and GABA $\beta$ R1a or GABA $\beta$ R1b.

Where binding of the substance or ligand is measured, such binding can be measured by employing a labeled substance or ligand. The substance or ligand can be labeled in any convenient manner known to the art, *e.g.*, radioactively, fluorescently,  
10 enzymatically.

In particular embodiments of the above-described methods, the substance or ligand is an amino acid or an amino acid analogue such as CGP71872, GABA, saclofen, (-)baclofen, glycine, and (L)-glutamic acid.

15 In particular embodiments of the above-described methods, HG20 has an amino acid sequence of SEQ.ID.NO.:2.

In particular embodiments of the above-described methods, HG20 comprises an amino acid sequence selected from the group consisting of:

20 SEQ.ID.NO.:2;  
Positions 9-941 of SEQ.ID.NO.:2;  
Positions 35-941 of SEQ.ID.NO.:2;  
Positions 36-941 of SEQ.ID.NO.:2;  
Positions 38-941 of SEQ.ID.NO.:2;  
25 Positions 39-941 of SEQ.ID.NO.:2;  
Positions 42-941 of SEQ.ID.NO.:2;  
Positions 44-941 of SEQ.ID.NO.:2;  
Positions 46-941 of SEQ.ID.NO.:2;  
Positions 52-941 of SEQ.ID.NO.:2; and  
30 Positions 57-941 of SEQ.ID.NO.:2.

In particular embodiments, GABA $\beta$ R1a is murine GABA $\beta$ R1a and has the amino acid sequence SEQ.ID.NO.:20. In particular embodiments, GABA $\beta$ R1a is rat GABA $\beta$ R1a and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-  
35 246. In particular embodiments, GABA $\beta$ R1b is rat GABA $\beta$ R1b and has the amino acid sequence reported in Kaupmann et al., 1997, Nature

386:239-246. In particular embodiments, GABA $\beta$ R1a is human GABA $\beta$ R1a and has an amino acid sequence selected from the group consisting of: SEQ.ID.NO.:21 and the protein encoded by SEQ.ID.NO.:23.

The above-described methods can be modified in that,  
5 rather than exposing cells to the substance, membranes can be prepared from the cells and those membranes can be exposed to the substance. Such a modification utilizing membranes rather than cells is well known in the art with respect to other receptors and is described in, *e.g.*, Hess et al., 1992, Biochem. Biophys. Res. Comm. 184:260-268.

10 As a further modification of the above-described method, RNA encoding HG20 and GABA $\beta$ R1a or GABA $\beta$ R1b can be prepared as, *e.g.*, by *in vitro* transcription using a plasmid containing HG20 and a plasmid containing GABA $\beta$ R1a or GABA $\beta$ R1b under the control of a bacteriophage T7 promoter, and the RNA can be microinjected into  
15 *Xenopus* oocytes in order to cause the expression of HG20 and GABA $\beta$ R1a or GABA $\beta$ R1b in the oocytes. Substances are then tested for binding to the heterodimer of HG20 and GABA $\beta$ R1a or GABA $\beta$ R1b expressed in the oocytes. Alternatively, rather than detecting binding, the effect of the substances on the electrophysiological properties of the  
20 oocytes can be determined.

The present invention includes assays by which GABA $\beta$  receptor agonists and antagonists may be identified by their ability to stimulate or antagonize a functional response mediated by the GABA $\beta$  receptor in cells that have been co-transfected with and that co-express  
25 HG20 and GABA $\beta$ R1a or GABA $\beta$ R1b.

Accordingly, the present invention provides a method of identifying agonists and antagonists of HG20 comprising:

- (a) providing test cells by transfecting cells with:
  - (1) an expression vector that directs the  
30 expression of HG20 in the cells; and
  - (2) an expression vector that directs the expression of GABA $\beta$ R1a or GABA $\beta$ R1b in the cells;
- (b) exposing the test cells to a substance that is suspected of being an agonist of the GABA $\beta$  receptor;
- 35 (c) measuring the amount of a functional response of the test cells that have been exposed to the substance;

(d) comparing the amount of the functional response exhibited by the test cells with the amount of the functional response exhibited by control cells;

5 wherein if the amount of the functional response exhibited by the test cells differs from the amount of the functional response exhibited by the control cells, the substance is an agonist or antagonist of the GABAB receptor;

10 where the control cells are cells that have not been transfected with HG20 and GABABR1a or GABABR1b but have been exposed to the substance or are test cells that have not been exposed to the substance.

In particular embodiments of the above-described methods, HG20 has an amino acid sequence of SEQ.ID.NO.:2.

15 In particular embodiments of the above-described methods, HG20 comprises an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;  
Positions 9-941 of SEQ.ID.NO.:2;  
Positions 35-941 of SEQ.ID.NO.:2;  
20 Positions 36-941 of SEQ.ID.NO.:2;  
Positions 38-941 of SEQ.ID.NO.:2;  
Positions 39-941 of SEQ.ID.NO.:2;  
Positions 42-941 of SEQ.ID.NO.:2;  
Positions 44-941 of SEQ.ID.NO.:2;  
25 Positions 46-941 of SEQ.ID.NO.:2;  
Positions 52-941 of SEQ.ID.NO.:2; and  
Positions 57-941 of SEQ.ID.NO.:2.

In particular embodiments, GABABR1a is murine GABABR1a and has the amino acid sequence SEQ.ID.NO.:20. In  
30 particular embodiments, GABABR1a is rat GABABR1a and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In particular embodiments, GABABR1b is rat GABABR1b and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In particular embodiments, GABABR1a is human  
35 GABABR1a and has an amino acid sequence selected from the group consisting of: SEQ.ID.NO.:21 and the protein encoded by SEQ.ID.NO.:23.

In particular embodiments, the functional response is selected from the group consisting of: changes in pigment distribution in melanophore cells; changes in cAMP or calcium concentration; and changes in membrane currents in *Xenopus* oocytes. In particular  
5       embodiments, the change in pigment distribution is pigment aggregation; the change in cAMP concentration is a decrease in cAMP concentration; the change in membrane current is the modulation of an inwardly rectifying potassium current.

In a particular embodiment of the above-described method,  
10       the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK-) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92),  
15       NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, or *Xenopus* oocytes.

In a particular embodiment of the above-described method, the cells are transfected with separate expression vectors that direct the  
20       expression of HG20 and either GABABR1a or GABABR1b in the cells. In other embodiments, the cells are transfected with a single expression vector that direct the expression of both HG20 and GABABR1a or GABABR1b in the cells.

In a particular embodiment, the cells are *Xenopus*  
25       melanophores and the functional response is pigment aggregation. In another embodiment, the cells are HEK293 cells and the functional response is a decrease in cAMP level. In another embodiment, the cells are *Xenopus* oocytes and the functional response is the production of an inwardly rectifying potassium current.

30       The conditions under which step (b) of the method is practiced are conditions that are typically used in the art for the study of protein-ligand interactions: e.g., physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

35       The above-described assay can be easily modified to form a method to identify antagonists of the GABAB receptor. Such a method comprises:

- (a) providing cells by transfecting cells with:
- (1) an expression vector that directs the expression of HG20 in the cells; and
  - (2) an expression vector that directs the expression of GABABR1a or GABABR1b in the cells;
- (b) exposing the cells to a substance that is a known agonist of the GABAB receptor;
- (c) measuring the amount of a functional response of the cells that have been exposed to the known agonist;
- (d) exposing the cells concurrently to the known agonist and to a substance that is suspected of being an antagonist of the GABAB receptor;
- (e) measuring the amount of a functional response of the cells that have been exposed to the substance and the known agonist;
- (f) comparing the amount of the functional response measured in step (c) with the amount of the functional response measured in step (e);
- wherein if the amount of the functional response measured in step (c) is greater than the amount of the functional response measured in step (e), the substance is an antagonist of the GABAB receptor.

Additional types of functional assays that can be used to identify agonists and antagonists of GABAB receptors include transcription-based assays. Transcription-based assays involve the use of a reporter gene whose transcription is driven by an inducible promoter whose activity is regulated by a particular intracellular event such as, *e.g.*, changes in intracellular calcium levels that are caused by the interaction of a receptor with a ligand. Transcription-based assays are reviewed in Rutter et al., 1998, *Chemistry & Biology* 5:R285-R290.

The transcription-based assays of the present invention rely on the expression of reporter genes whose transcription is activated or repressed as a result of intracellular events that are caused by the interaction of an agonist with a heterodimer of HG20 and either GABABR1a or GABABR1b where the heterodimer forms a functional GABAB receptor.

An extremely sensitive transcription based assay is disclosed in Zlokarnik et al., 1998, *Science* 279:84-88 (Zlokarnik) and also

in U.S. Patent No. 5,741,657. The assay disclosed in Zlokarnik and U.S. Patent No. 5,741,657 employs a plasmid encoding  $\beta$ -lactamase under the control of an inducible promoter. This plasmid is transfected into cells together with a plasmid encoding a receptor for which it is desired to  
5 identify agonists. The inducible promoter on the  $\beta$ -lactamase is chosen so that it responds to at least one intracellular signal that is generated when an agonist binds to the receptor. Thus, following such binding of agonist to receptor, the level of  $\beta$ -lactamase in the transfected cells increases. This increase in  $\beta$ -lactamase is made measurable by treating  
10 the cells with a cell-permeable dye that is a substrate for  $\beta$ -lactamase. The dye contains two fluorescent moieties. In the intact dye, the two fluorescent moieties are close enough to one another that fluorescent resonance energy transfer (FRET) can take place between them. Following cleavage of the dye into two parts by  $\beta$ -lactamase, the two  
15 fluorescent moieties are located on different parts, and thus can drift apart. This increases the distance between the fluorescent moieties, thus decreasing the amount of FRET that can occur between them. It is this decrease in FRET that is measured in the assay.

One skilled in the art can modify the assay described in  
20 Zlokarnik and U.S. Patent No. 5,741,657 to form an assay for identifying agonists of GABA<sub>B</sub> receptors by using an inducible promoter to drive  $\beta$ -lactamase that is activated by an intracellular signal generated by the interaction of agonists and the GABA<sub>B</sub> receptor. To produce the GABA<sub>B</sub> receptor, a plasmid encoding HG20 and a plasmid encoding  
25 GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b would be transfected into the cells. The cells would be exposed to the cell-permeable dye and then exposed to substances suspected of being agonists of the GABA<sub>B</sub> receptor. Those substances that cause a decrease in FRET are likely to actually be agonists of the GABA<sub>B</sub> receptor.

30 Accordingly, the present invention includes a method for identifying agonists of the GABA<sub>B</sub> receptor comprising:

- (a) transfecting cells with:
  - (1) an expression vector that directs the  
expression of HG20 in the cells;
  - 35 (2) an expression vector that directs the  
expression of GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the cells;

- (3) an expression vector that directs the expression of  $\beta$ -lactamase under the control of an inducible promoter that is activated by an intracellular signal generated by the interaction of agonists and the GABA<sub>B</sub> receptor;
- 5 (b) exposing the cells to a substrate of  $\beta$ -lactamase that is a cell-permeable dye that contains two fluorescent moieties where the two fluorescent moieties are on different parts of the dye and cleavage of the dye by  $\beta$ -lactamase allows the two fluorescent moieties to drift apart;
- 10 (c) measuring the amount of fluorescent resonance energy transfer (FRET) in the cells in the absence of the substance of step (d);
- (d) exposing the cells to a substance that is suspected of being an agonist of the GABA<sub>B</sub> receptor;
- 15 (e) measuring the amount of FRET in the cells after exposure of the cells to the substance;
- wherein if the amount of FRET in the cells measured in step (e) is less than the amount of FRET measured in the cells in step (c), then the substance is an agonist of the GABA<sub>B</sub> receptor.
- 20 Substeps (1)-(3) of step (a) can be practiced in any order.
- The assay described above can be modified to an assay for identifying antagonists of the GABA<sub>B</sub> receptor. Such modification would involve the use of  $\beta$ -lactamase under the control of a promoter that is repressed by at least one intracellular signal generated by
- 25 interaction of an agonist with the GABA<sub>B</sub> receptor and would also involve running the assay in the presence of a known agonist. When the cells are exposed to substances suspected of being antagonists of the GABA<sub>B</sub> receptor,  $\beta$ -lactamase will be induced, and FRET will decrease, only if the substance tested is able to counteract the effect of the agonist,
- 30 i.e., only if the substance tested is actually an antagonist.
- Accordingly, the present invention includes a method for identifying antagonists of the GABA<sub>B</sub> receptor comprising:
- (a) transfecting cells with:
- (1) an expression vector that directs the
- 35 expression of HG20 in the cells;
- (2) an expression vector that directs the expression of GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the cells;

- (3) an expression vector that directs the expression of  $\beta$ -lactamase under the control of an inducible promoter that is repressed by at least one intracellular signal generated by interaction of an agonist with the GABA<sub>B</sub> receptor;
- 5 (b) exposing the cells to a known agonist of the GABA<sub>B</sub> receptor;
- (c) exposing the cells to a substrate of  $\beta$ -lactamase that is a cell-permeable dye that contains two fluorescent moieties where the two fluorescent moieties are on different parts of the dye and cleavage of
- 10 the dye by  $\beta$ -lactamase allows the two fluorescent moieties to drift apart;
- (d) measuring the amount of fluorescent resonance energy transfer (FRET) in the cells in the absence of the substance of step (e);
- 15 (e) exposing the cells to a substance that is suspected of being an antagonist of the GABA<sub>B</sub> receptor;
- (f) measuring the amount of FRET in the cells after exposure of the cells to the substance;
- wherein if the amount of FRET in the cells measured in
- 20 step (f) is less than the amount of FRET measured in the cells in step (d), then the substance is an antagonist of the GABA<sub>B</sub> receptor.
- Substeps (1)-(3) of step (a) can be practiced in any order.
- In particular embodiments of the assays employing  $\beta$ -lactamase described above, the cells are eukaryotic cells. In particular
- 25 embodiments, the cells are mammalian cells. In particular embodiments, the cells are selected from the group consisting of: L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC
- 30 CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, and *Xenopus* oocytes.
- In other embodiments, the inducible promoter that is repressed by at least one intracellular signal generated by interaction of
- 35 an agonist with the GABA<sub>B</sub> receptor is a promoter that is repressed by decreases in cAMP levels or changes in potassium currents.



In other embodiments, the inducible promoter that is activated by at least one intracellular signal generated by interaction of an agonist with the GABAB receptor is a promoter that is activated by decreases in cAMP levels or changes in potassium currents.

5 In other embodiments, the known agonist is selected from the group consisting of: GABA, saclofen, (-)baclofen, glycine, and (L)-glutamic acid.

In other embodiments,  $\beta$ -lactamase is TEM-1  $\beta$ -lactamase from *Escherichia coli*.

10 In other embodiments, the substrate of  $\beta$ -lactamase is CCF2/AM (Zlokarnik et al., 1998, Science 279:84-88).

In other embodiments, HG20 has an amino acid sequence of SEQ.ID.NO.:2.

15 In other embodiments of the above-described methods, HG20 comprises an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;

Positions 9-941 of SEQ.ID.NO.:2;

Positions 35-941 of SEQ.ID.NO.:2;

20 Positions 36-941 of SEQ.ID.NO.:2;

Positions 38-941 of SEQ.ID.NO.:2;

Positions 39-941 of SEQ.ID.NO.:2;

Positions 42-941 of SEQ.ID.NO.:2;

Positions 44-941 of SEQ.ID.NO.:2;

25 Positions 46-941 of SEQ.ID.NO.:2;

Positions 52-941 of SEQ.ID.NO.:2; and

Positions 57-941 of SEQ.ID.NO.:2.

In other embodiments, GABABR1a is murine GABABR1a and has the amino acid sequence SEQ.ID.NO.:20. In other  
30 embodiments, GABABR1a is rat GABABR1a and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In other embodiments, GABABR1b is rat GABABR1b and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In other embodiments, GABABR1a is human GABABR1a and has an  
35 amino acid sequence selected from the group consisting of: SEQ.ID.NO.:21 and the protein encoded by SEQ.ID.NO.:23.

In particular embodiments, the cells express a promiscuous G-protein, *e.g.*, G $\alpha$ 15 or G $\alpha$ 16.

In particular embodiments, the inducible promoter is a promoter that is activated or repressed by NF- $\kappa$ B or NFAT.

5           The assays described above could be modified to identify inverse agonists. In such assays, one would expect a decrease in  $\beta$ -lactamase activity. Similarly, inverse agonists can be identified by modifying the functional assays that were described previously where those functional assays monitored decreases in cAMP levels. In the  
10       case of assays for inverse agonists, increases in cAMP levels would be observed.

Other transcription-based assays that can be used to identify agonists and antagonists of the GABA $\beta$  receptor rely on the use of green fluorescent proteins or luciferase as reported genes. An  
15       example of such an assay comprises:

- (a)   transfecting cells with:
  - (1)   an expression vector that directs the expression of HG20 in the cells;
  - (2)   an expression vector that directs the  
20       expression of GABA $\beta$ R1a or GABA $\beta$ R1b in the cells;
  - (3)   an expression vector that directs the expression of green fluorescent protein (GFP) under the control of an inducible promoter that is activated by an intracellular signal generated by the interaction of agonists and the GABA $\beta$  receptor;
- 25       (b)   measuring the amount of fluorescence from GFP in the cells;
- (c)   exposing the cells to a substance that is suspected of being an agonist of the GABA $\beta$  receptor;
- (d)   measuring the amount of fluorescence from GFP in  
30       the cells that have been exposed to the substance;
- wherein if the amount of fluorescence from GFP in the cells measured in step (b) is less than the amount of fluorescence from GFP measured in the cells in step (d), then the substance is an agonist of the GABA $\beta$  receptor.

35           The present invention also includes assays for the identification of agonists or antagonists of GABA $\beta$  receptors that are based upon FRET between a first and a second fluorescent dye where the

first dye is bound to one side of the plasma membrane of a cell expressing a heterodimer of HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b and the second dye is free to shuttle from one face of the membrane to the other face in response to changes in membrane potential. In certain  
5   embodiments, the first dye is impenetrable to the plasma membrane of the cells and is bound predominately to the extracellular surface of the plasma membrane. The second dye is trapped within the plasma membrane but is free to diffuse within the membrane. At normal (*i.e.*, negative) resting potentials of the membrane, the second dye is bound  
10   predominately to the inner surface of the extracellular face of the plasma membrane, thus placing the second dye in close proximity to the first dye. This close proximity allows for the generation of a large amount of FRET between the two dyes. Following membrane depolarization, the second dye moves from the extracellular face of the  
15   membrane to the intracellular face, thus increasing the distance between the dyes. This increased distance results in a decrease in FRET, with a corresponding increase in fluorescent emission derived from the first dye and a corresponding decrease in the fluorescent emission from the second dye. See figure 1 of González & Tsien, 1997,  
20   Chemistry & Biology 4:269-277. See also González & Tsien, 1995, Biophys. J. 69:1272-1280 and U.S. Patent No. 5,661,035.

          In certain embodiments, the first dye is a fluorescent lectin or a fluorescent phospholipid that acts as the fluorescent donor. Examples of such a first dye are: a coumarin-labeled  
25   phosphatidylethanolamine (*e.g.*, N-(6-chloro-7-hydroxy-2-oxo-2H--1-benzopyran-3-carboxamidoacetyl)-dimyristoylphosphatidyl-ethanolamine) or N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-dipalmitoylphosphatidylethanolamine); a fluorescently-labeled lectin (*e.g.*, fluorescein-labeled wheat germ agglutinin). In certain  
30   embodiments, the second dye is an oxonol that acts as the fluorescent acceptor. Examples of such a second dye are: bis(1,3-dialkyl-2-thiobarbiturate)trimethineoxonols (*e.g.*, bis(1,3-dihexyl-2-thiobarbiturate)trimethineoxonol) or pentamethineoxonol analogues (*e.g.*, bis(1,3-dihexyl-2-thiobarbiturate)pentamethineoxonol; or bis(1,3-  
35   dibutyl-2-thiobarbiturate)pentamethineoxonol). See González & Tsien, 1997, Chemistry & Biology 4:269-277 for methods of synthesizing various dyes suitable for use in the present invention. In certain embodiments,

the assay may comprise a natural carotenoid, *e.g.*, astaxanthin, in order to reduce photodynamic damage due to singlet oxygen.

Accordingly, the present invention provides a method of identifying agonists of GABA<sub>B</sub> receptors comprising:

- 5 (a) providing test cells comprising:
    - (1) an expression vector that directs the expression of HG20 in the cells;
    - (2) an expression vector that directs the expression of GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the cells;
    - 10 (3) an expression vector that directs the expression of an inwardly rectifying potassium channel;
    - (4) a first fluorescent dye, where the first dye is bound to one side of the plasma membrane; and
    - (5) a second fluorescent dye, where the second
    - 15 fluorescent dye is free to shuttle from one face of the plasma membrane to the other face in response to changes in membrane potential;
  - (b) exposing the test cells to a substance that is suspected of being an agonist of the GABA<sub>B</sub> receptor;
  - (c) measuring the amount of fluorescence resonance
  - 20 energy transfer (FRET) in the test cells that have been exposed to the substance;
  - (d) comparing the amount of FRET exhibited by the test cells that have been exposed to the substance with the amount of FRET exhibited by control cells;
  - 25 wherein if the amount of FRET exhibited by the test cells is less than the amount of FRET exhibited by the control cells, the substance is an agonist of the GABA<sub>B</sub> receptor;
  - where the control cells are either (1) cells that are essentially the same as the test cells except that they do not comprise at
  - 30 least one of the items listed at (a) (1)-(5) but have been exposed to the substance; or (2) test cells that have not been exposed to the substance.
- The above-described assay can be easily modified to form a method to identify antagonists of the GABA<sub>B</sub> receptor. Such a method comprises:
- 35 (a) providing test cells comprising:
    - (1) an expression vector that directs the expression of HG20 in the cells;

- (2) an expression vector that directs the expression of GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the cells;
- (3) an expression vector that directs the expression of an inwardly rectifying potassium channel;
- 5 (4) a first fluorescent dye, where the first dye is bound to one side of the plasma membrane; and
- (5) a second fluorescent dye, where the second fluorescent dye is free to shuttle from one face of the plasma membrane to the other face in response to changes in membrane potential;
- 10 (b) exposing the test cells to a known agonist of the GABA<sub>B</sub> receptor in the presence of a substance that is suspected of being an antagonist of the GABA<sub>B</sub> receptor;
- (c) exposing the test cells to the known agonist of the GABA<sub>B</sub> receptor in the absence of the substance that is suspected of being
- 15 an antagonist of the GABA<sub>B</sub> receptor;
- (d) measuring the amount of fluorescence resonance energy transfer (FRET) in the test cells of steps (b) and (c);
- (e) comparing the amount of FRET exhibited by the test cells of steps (b) and (c);
- 20 where if the amount of FRET exhibited by the test cells of step (b) is greater than the amount of FRET exhibited by the test cells of step (c), the substance is an antagonist of the GABA<sub>B</sub> receptor.
- In particular embodiments of the above-described methods, the expression vectors are transfected into the test cells.
- 25 In particular embodiments of the above-described methods, HG20 has an amino acid sequence of SEQ.ID.NO.:2.
- In particular embodiments of the above-described methods, HG20 comprises an amino acid sequence selected from the group consisting of:
- 30 SEQ.ID.NO.:2;  
Positions 9-941 of SEQ.ID.NO.:2;  
Positions 35-941 of SEQ.ID.NO.:2;  
Positions 36-941 of SEQ.ID.NO.:2;  
Positions 38-941 of SEQ.ID.NO.:2;  
35 Positions 39-941 of SEQ.ID.NO.:2;  
Positions 42-941 of SEQ.ID.NO.:2;  
Positions 44-941 of SEQ.ID.NO.:2;

Positions 46-941 of SEQ.ID.NO.:2;  
Positions 52-941 of SEQ.ID.NO.:2; and  
Positions 57-941 of SEQ.ID.NO.:2.

In particular embodiments of the above-described methods,  
5 GABABR1a is murine GABABR1a and has the amino acid sequence  
SEQ.ID.NO.:20. In particular embodiments, GABABR1a is rat  
GABABR1a and has the amino acid sequence reported in Kaupmann et  
al., 1997, Nature 386:239-246. In particular embodiments, GABABR1b is  
10 rat GABABR1b and has the amino acid sequence reported in Kaupmann  
et al., 1997, Nature 386:239-246. In particular embodiments, GABABR1a  
is human GABABR1a and has an amino acid sequence selected from  
the group consisting of: SEQ.ID.NO.:21 and the protein encoded by  
SEQ.ID.NO.:23.

Inwardly rectifying potassium channels that are suitable  
15 for use in the methods of the present invention are disclosed in, *e.g.*,  
Misgeld et al., 1995, Prog. Neurobiol. 46:423-462; North, 1989, Br. J.  
Pharmacol. 98:13-23; Gahwiler et al., 1985, Proc. Natl. Acad. Sci USA  
82:1558-1562; Andrade et al., 1986, Science 234:1261.

In particular embodiments of the above-described methods,  
20 the first fluorescent dye is selected from the group consisting of: a  
fluorescent lectin; a fluorescent phospholipid; a coumarin-labeled  
phosphatidylethanolamine; N-(6-chloro-7-hydroxy-2-oxo-2H--1-  
benzopyran-3-carboxamidoacetyl)-dimyristoylphosphatidyl-  
ethanolamine); N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-  
25 dipalmitoylphosphatidylethanolamine); and fluorescein-labeled wheat  
germ agglutinin.

In particular embodiments of the above-described methods,  
the second fluorescent dye is selected from the group consisting of: an  
oxonol that acts as the fluorescent acceptor; bis(1,3-dialkyl-2-  
30 thiobarbiturate)trimethineoxonols; bis(1,3-dihexyl-2-  
thiobarbiturate)trimethineoxonol; bis(1,3-dialkyl-2-  
thiobarbiturate)quatramethineoxonols; bis(1,3-dialkyl-2-  
thiobarbiturate)pentamethineoxonols; bis(1,3-dihexyl-2-  
thiobarbiturate)pentamethineoxonol; bis(1,3-dibutyl-2-  
35 thiobarbiturate)pentamethineoxonol); and bis(1,3-dialkyl-2-  
thiobarbiturate)hexamethineoxonols.

In a particular embodiment of the above-described methods, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573),  
5 Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, or *Xenopus* oocytes.

10 In a particular embodiment of the above-described methods, the cells are transfected with separate expression vectors that direct the expression of HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the cells. In other embodiments, the cells are transfected with a single expression vector that direct the expression of both HG20 and GABA<sub>B</sub>R1a or  
15 GABA<sub>B</sub>R1b in the cells.

The conditions under which step (b) of the first method described above and steps (b) and (c) of the second method described above are practiced are conditions that are typically used in the art for the study of protein-ligand interactions: *e.g.*, physiological pH; salt  
20 conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

The GABA<sub>B</sub> receptor belongs to the class of proteins known as G-protein coupled receptors (GPCRs). GPCRs transmit signals across cell membranes upon the binding of ligand. The ligand-bound  
25 GPCR interacts with a heterotrimeric G-protein, causing the G $\alpha$  subunit of the G-protein to disassociate from the G $\beta$  and G $\gamma$  subunits. The G $\alpha$  subunit can then go on to activate a variety of second messenger systems.

Generally, a particular GPCR is only coupled to a  
30 particular type of G-protein. Thus, to observe a functional response from the GPCR, it is necessary to ensure that the proper G-protein is present in the system containing the GPCR. It has been found, however, that there are certain G-proteins that are "promiscuous." These promiscuous G-proteins will couple to, and thus transduce a  
35 functional signal from, virtually any GPCR. See Offermanns & Simon, 1995, J. Biol. Chem. 270:15175, 15180 (Offermanns). Offermanns described a system in which cells are transfected with expression

vectors that result in the expression of one of a large number of GPCRs as well as the expression of one of the promiscuous G-proteins  $G\alpha 15$  or  $G\alpha 16$ . Upon the addition of an agonist of the GPCR to the transfected cells, the GPCR was activated and was able, via  $G\alpha 15$  or  $G\alpha 16$ , to  
5 activate the  $\beta$  isoform of phospholipase C, leading to an increase in inositol phosphate levels in the cells.

Therefore, by making use of these promiscuous G-proteins as in Offermanns, it is possible to set up functional assays for the GABA<sub>B</sub> receptor, even in the absence of knowledge of the G-protein with which  
10 the GABA<sub>B</sub> receptor is coupled *in vivo*. One possibility for utilizing promiscuous G-proteins in connection with the GABA<sub>B</sub> receptor includes a method of identifying agonists of the GABA<sub>B</sub> receptor comprising:

- (a) providing cells that express HG20, GABA<sub>B</sub>R1a or  
15 GABA<sub>B</sub>R1b, and a promiscuous G-protein, where HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b form a heterodimer representing a functional GABA<sub>B</sub> receptor;
- (b) exposing the cells to a substance that is a suspected agonist of the GABA<sub>B</sub> receptor;
- 20 (c) measuring the level of inositol phosphates in the cells;

where an increase in the level of inositol phosphates in the cells as compared to the level of inositol phosphates in the cells in the absence of the suspected agonist indicates that the substance is an  
25 agonist of the GABA<sub>B</sub> receptor.

Levels of inositol phosphates can be measured by monitoring calcium mobilization. Intracellular calcium mobilization is typically assayed in whole cells under a microscope using fluorescent dyes or in cell suspensions via luminescence using the aequorin assay.

30 In methods related to those described above, rather than using changes in inositol phosphate levels as an indication of GABA<sub>B</sub> receptor function, potassium currents are measured. This is feasible since the GABA<sub>B</sub> receptor, like other metabotropic receptors, is expected to be coupled to potassium channels. Thus, one could measure GABA<sub>B</sub>  
35 receptor coupling to GIRK2 channels or to other potassium channels in oocytes.



In a particular embodiment of the above-described method, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573),  
5 Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), or *Xenopus* oocytes.

10 In a particular embodiment of the above-described method, the cells are transfected with expression vectors that direct the expression of HG20, GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, and the promiscuous G-protein in the cells.

The conditions under which step (b) of the method is  
15 practiced are conditions that are typically used in the art for the study of protein-ligand interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In a particular embodiment of the above-described method,  
20 the promiscuous G-protein is selected from the group consisting of Gα15 or Gα16. Expression vectors containing Gα15 or Gα16 are known in the art. See, *e.g.*, Offermanns; Buhl et al, 1993, FEBS Lett. 323:132-134; Amatruda et al., 1993, J. Biol. Chem. 268:10139-10144.

The above-described assay can be easily modified to form a  
25 method to identify antagonists of the GABA<sub>B</sub> receptor. Such a method is also part of the present invention and comprises:

- (a) providing cells that express HG20, GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, and a promiscuous G-protein;
- (b) exposing the cells to a substance that is an agonist of  
30 the GABA<sub>B</sub> receptor;
- (c) subsequently or concurrently to step (b), exposing the cells to a substance that is a suspected antagonist of the GABA<sub>B</sub> receptor;
- (d) measuring the level of inositol phosphates in the  
cells;

35 where a decrease in the level of inositol phosphates in the cells in the presence of the suspected antagonist as compared to the level of inositol phosphates in the cells in the absence of the suspected

antagonist indicates that the substance is an antagonist of the GABA<sub>B</sub> receptor.

In a particular embodiment of the above-described method, the agonist is an amino acid such as GABA, glutamate, glycine, or  
5 amino acid analogues such as (-)baclofen.

In a particular embodiment of the above-described method, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK-) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL  
10 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), or Xenopus oocytes.

15 The conditions under which steps (b) and (c) of the method are practiced are conditions that are typically used in the art for the study of protein-ligand interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

20 In a particular embodiment of the above-described method, the cells are transfected with expression vectors that direct the expression of HG20, GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, and the promiscuous G-protein in the cells.

In a particular embodiment of the above-described method,  
25 the promiscuous G-protein is selected from the group consisting of Gα15 or Gα16.

In particular embodiments of the above-described methods, HG20 has an amino acid sequence of SEQ.ID.NO.:2.

In other embodiments of the above-described methods,  
30 HG20 comprises an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;  
Positions 9-941 of SEQ.ID.NO.:2;  
Positions 35-941 of SEQ.ID.NO.:2;  
35 Positions 36-941 of SEQ.ID.NO.:2;  
Positions 38-941 of SEQ.ID.NO.:2;

Positions 39-941 of SEQ.ID.NO.:2;  
Positions 42-941 of SEQ.ID.NO.:2;  
Positions 44-941 of SEQ.ID.NO.:2;  
Positions 46-941 of SEQ.ID.NO.:2;  
5 Positions 52-941 of SEQ.ID.NO.:2; and  
Positions 57-941 of SEQ.ID.NO.:2.

In other embodiments, GABA<sub>B</sub>R1a is murine GABA<sub>B</sub>R1a and has the amino acid sequence SEQ.ID.NO.:20. In other  
embodiments, GABA<sub>B</sub>R1a is rat GABA<sub>B</sub>R1a and has the amino acid  
10 sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In  
other embodiments, GABA<sub>B</sub>R1b is rat GABA<sub>B</sub>R1b and has the amino  
acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In  
other embodiments, GABA<sub>B</sub>R1a is human GABA<sub>B</sub>R1a and has an  
amino acid sequence selected from the group consisting of:  
15 SEQ.ID.NO.:21 and the protein encoded by SEQ.ID.NO.:23.

While the above-described methods are explicitly directed to  
testing whether "a" substance is an agonist or antagonist of the GABA<sub>B</sub>  
receptor, it will be clear to one skilled in the art that such methods can  
be adapted to test collections of substances, *e.g.*, combinatorial libraries,  
20 to determine whether any members of such collections are activators or  
inhibitors of the GABA<sub>B</sub> receptor. Accordingly, the use of collections of  
substances, or individual members of such collections, as the substance  
in the above-described methods is within the scope of the present  
invention.

25 The present invention includes pharmaceutical  
compositions comprising agonists and antagonists of GABA<sub>B</sub> receptors  
that have been identified by the above-described methods. The agonists  
and antagonists are generally combined with pharmaceutically  
acceptable carriers to form pharmaceutical compositions. Examples of  
30 such carriers and methods of formulation of pharmaceutical  
compositions containing agonists and antagonists and carriers can be  
found in Remington's Pharmaceutical Sciences. To form a  
pharmaceutically acceptable composition suitable for effective  
administration, such compositions will contain a therapeutically  
35 effective amount of the agonists and antagonists.

Therapeutic or prophylactic compositions are administered  
to an individual in amounts sufficient to treat or prevent conditions

where GABA<sub>B</sub> receptor activity is abnormal. The effective amount can vary according to a variety of factors such as the individual's condition, weight, gender, and age. Other factors include the mode of administration. The appropriate amount can be determined by a skilled  
5 physician.

Compositions can be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents can be desirable.

The compositions can be administered in a wide variety of  
10 therapeutic dosage forms in conventional vehicles for administration. For example, the compositions can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection.  
15 Likewise, they can also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

Advantageously, compositions can be administered in a  
20 single daily dose, or the total daily dosage can be administered in divided doses of two, three or four times daily. Furthermore, compositions can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be  
25 administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

The dosage regimen utilizing the compositions is selected in accordance with a variety of factors including type, species, age,  
30 weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal, hepatic and cardiovascular function of the patient; and the particular composition thereof employed. A physician of ordinary skill can readily determine and prescribe the effective amount of the composition  
35 required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of composition within

the range that yields efficacy without toxicity requires a regimen based on the kinetics of the composition's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a composition.

- 5                   Agonists and antagonists identified by the above-described methods are useful in the same manner as well-known agonists and antagonists of other GABA<sub>B</sub> receptors. For example, (-) baclofen is a known agonist of GABA<sub>B</sub> receptors and, in racemic form, is a clinically useful muscle relaxant known as LIORESAL® (Bowery & Pratt, 1992, 10 *Arzneim.-Forsch./Drug Res.* 42:215-223 [Bowery & Pratt]). Similarly, the agonists and antagonists of GABA<sub>B</sub> receptors identified by the methods of the present invention are expected to be useful as muscle relaxants. Bowery & Pratt, at Table 1, page 219, list the therapeutic potential of GABA<sub>B</sub> receptor agonists and antagonists. For agonists, the 15 therapeutic potential is said to include use as muscle relaxants and anti-asthmatics. For antagonists, the therapeutic potential is said to include use as antidepressants, anticonvulsants, nootropics, and anxiolytics. Additionally, at page 220, left column, Bowery & Pratt list some additional therapeutic uses for the GABA<sub>B</sub> receptor agonist (-) 20 baclofen: treatment of trigeminal neuralgia and reversal of ethanol withdrawal symptoms. Given the wide range of utility displayed by known agonists and antagonists of GABA<sub>B</sub> receptors, it is clear that those skilled in the art would consider the agonists and antagonists identified by the methods of the present invention to be 25 pharmacologically useful. In addition, it is believed that such agonists and antagonists will also be useful in the treatment of epilepsy, neuropsychiatric disorders, and dementias.

- When screening compounds in order to identify potential pharmaceuticals that specifically interact with a target receptor, it is 30 necessary to ensure that the compounds identified are as specific as possible for the target receptor. To do this, it is necessary to screen the compounds against as wide an array as possible of receptors that are similar to the target receptor. Thus, in order to find compounds that are potential pharmaceuticals that interact with receptor A, it is necessary 35 not only to ensure that the compounds interact with receptor A (the "plus target") and produce the desired pharmacological effect through receptor A, it is also necessary to determine that the compounds do not

interact with receptors B, C, D, etc (the "minus targets"). In general, as part of a screening program, it is important to have as many minus targets as possible (see Hodgson, 1992, Bio/Technology 10:973-980, at 980). HG20 protein, DNA encoding HG20 protein, GABABR1a protein, DNA  
5 encoding GABABR1a protein, and recombinant cells that have been engineered to express HG20 protein and GABABR1a protein have utility in that they can be used as "minus targets" in screens design to identify compounds that specifically interact with other G-protein coupled receptors, *i.e.*, non-GABAB receptors.

10 The present invention also includes antibodies to the HG20 protein. Such antibodies may be polyclonal antibodies or monoclonal antibodies. The antibodies of the present invention are raised against the entire HG20 protein or against suitable antigenic fragments of the protein that are coupled to suitable carriers, *e.g.*, serum albumin or  
15 keyhole limpet hemocyanin, by methods well known in the art. Methods of identifying suitable antigenic fragments of a protein are known in the art. See, *e.g.*, Hopp & Woods, 1981, Proc. Natl. Acad. Sci. USA 78:3824-3828; and Jameson & Wolf, 1988, CABIOS (Computer Applications in the Biosciences) 4:181-186. Particularly suitable peptides are: amino acids  
20 357-371 of SEQ.ID.NO.:2 and amino acids 495-511 of SEQ.ID.NO.:2. Also, anti-peptide antisera can be generated by immunization of New Zealand White rabbits with a KLH-conjugation of a 20 amino acid synthetic peptide corresponding to residues 283-302 of HG20 (GWYEPSWWEQVHTEANSSRC) (a portion of SEQ.ID.NO.:2).

25 For the production of polyclonal antibodies, HG20 protein or an antigenic fragment, coupled to a suitable carrier, is injected on a periodic basis into an appropriate non-human host animal such as, *e.g.*, rabbits, sheep, goats, rats, mice. The animals are bled periodically and sera obtained are tested for the presence of antibodies to the injected  
30 antigen. The injections can be intramuscular, intraperitoneal, subcutaneous, and the like, and can be accompanied with adjuvant.

For the production of monoclonal antibodies, HG20 protein or an antigenic fragment, coupled to a suitable carrier, is injected into an appropriate non-human host animal as above for the production of  
35 polyclonal antibodies. In the case of monoclonal antibodies, the animal is generally a mouse. The animal's spleen cells are then immortalized, often by fusion with a myeloma cell, as described in Kohler & Milstein,

1975, Nature 256:495-497. For a fuller description of the production of monoclonal antibodies, see Antibodies: A Laboratory Manual, Harlow & Lane, eds., Cold Spring Harbor Laboratory Press, 1988.

Gene therapy may be used to introduce HG20 polypeptides into the cells of target organs. Nucleotides encoding HG20 polypeptides can be ligated into viral vectors which mediate transfer of the nucleotides by infection of recipient cells. Suitable viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, and polio virus based vectors. Alternatively, nucleotides encoding HG20 polypeptides can be transferred into cells for gene therapy by non-viral techniques including receptor-mediated targeted transfer using ligand-nucleotide conjugates, lipofection, membrane fusion, or direct microinjection. These procedures and variations thereof are suitable for *ex vivo* as well as *in vivo* gene therapy. Gene therapy with HG20 polypeptides will be particularly useful for the treatment of diseases where it is beneficial to elevate HG20 activity.

The following non-limiting examples are presented to better illustrate the invention.

20

### EXAMPLE 1

#### Cloning and sequencing of HG20

A cDNA fragment encoding full-length HG20 can be isolated from a human fetal brain cDNA library by using the polymerase chain reaction (PCR) employing the following primer pair:

25

HG20.F139      5'-CCGTTCTGAGCCGAGCCG -3' (SEQ.ID.NO.:3)  
HG20.R3195      5'-TCCGCAGCCAGAGCCGACAG-3' (SEQ.ID.NO.:4)

The above primer pair is meant to be illustrative only. Those skilled in the art would recognize that a large number of primer pairs, based upon SEQ.ID.NO.:1, could also be used.

PCR reactions can be carried out with a variety of thermostable enzymes including but not limited to AmpliTaq, AmpliTaq Gold, Vent polymerase. For AmpliTaq, reactions can be carried out in 10 mM Tris-Cl, pH 8.3, 2.0 mM MgCl<sub>2</sub>, 200 μM for each dNTP, 50 mM

KCl, 0.2  $\mu$ M for each primer, 10 ng of DNA template, 0.05 units/ $\mu$ l of AmpliTaq. The reactions are heated at 95°C for 3 minutes and then cycled 35 times using the cycling parameters of 95°C, 20 seconds, 62°C, 20 seconds, 72°C, 3 minutes. In addition to these conditions, a variety of  
5 suitable PCR protocols can be found in PCR Primer, A Laboratory Manual, edited by C.W. Dieffenbach and G.S. Dveksler, 1995, Cold Spring Harbor Laboratory Press.

A suitable cDNA library from which a clone encoding HG20 can be isolated would be a random primed fetal brain cDNA library  
10 consisting of approximately 4.0 million primary clones constructed in the plasmid vector pBluescript (Stratagene, LaJolla, CA). The primary clones of such a library can be subdivided into pools with each pool containing approximately 20,000 clones and each pool can be amplified separately.

By this method, a cDNA fragment (SEQ.ID.NO.:1) encoding an open reading frame of 941 amino acids (SEQ.ID.NO.:2) is obtained. This cDNA fragment can be cloned into a suitable cloning vector or expression vector. For example, the fragment can be cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, San Diego, CA).  
20 HG20 protein can then be produced by transferring an expression vector containing SEQ.ID.NO.:1 or portions thereof into a suitable host cell and growing the host cell under appropriate conditions. HG20 protein can then be isolated by methods well known in the art.

Alternatively, other cDNA libraries made from human  
25 tissues that express HG20 RNA can be used with PCR primers HG20.F139 and HG20.R3195 in order to amplify a cDNA fragment encoding full-length HG20. Suitable cDNA libraries would be those prepared from cortex, cerebellum, testis, ovary, adrenal gland, thyroid, or spinal cord.

As an alternative to the above-described PCR method, a  
30 cDNA clone encoding HG20 can be isolated from a cDNA library using as a probe oligonucleotides specific for HG20 and methods well known in the art for screening cDNA libraries with oligonucleotide probes. Such methods are described in, e.g., Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory, Cold  
35 Spring Harbor, New York; Glover, D.M. (ed.), 1985, *DNA Cloning: A Practical Approach*, MRL Press, Ltd., Oxford, U.K., Vol. I, II.



Oligonucleotides that are specific for HG20 and that can be used to screen cDNA libraries are:

	HG20.F46	5'-GGGATGATCATGGCCAGTGC-3' (SEQ.ID.NO.:5)
5	HG20.R179	5'-GGATCCATCAAGGCCAAAGA-3' (SEQ.ID.NO.:6)
	HG21.F43	5'-GCCGCTGTCTCCTTCCTGA-3' (SEQ.ID.NO.:7)
	HG21.R251	5'-TTGGTTCACACTGGTGACCGA-3' (SEQ.ID.NO.:8)
	HG20.R123	5'-TTCACCTCCCTGCTGTCTTG-3' (SEQ.ID.NO.:9)
	HG20.F1100	5'-CAGGCGATTCCAGTTCACCTCA-5' (SEQ.ID.NO.:10)
10	HG20.F1747	5'-GAACCAAGCCAGCACATCCC-3' (SEQ.ID.NO.:11)
	HG20.R54	5'-CCTCGCCATACAGAACTCC-3' (SEQ.ID.NO.:12)
	HG20.R75	5'-GTGTCATAGAGCCGCAGGTC-3' (SEQ.ID.NO.:13)
	HG20.F139	5'-CCGTTCTGAGCCGAGCCG-3' (SEQ.ID.NO.:3)
	HG20.R3195	5'-TCCGCAGCCAGAGCCGACAG-3' (SEQ.ID.NO.:4)

15

Membrane-spanning proteins, such as GABA<sub>B</sub> receptors, when first translated generally possess an approximately 16 to 40 amino acid segment known as a signal sequence. Signal sequences direct the nascent protein to be transported through the endoplasmic reticulum membrane, following which signal sequences are cleaved from the protein. Signal sequences generally contain from 4 to 12 hydrophobic residues but otherwise possess little sequence homology. The Protein Analysis tool of the GCG program (Genetics Computer Group, Madison, Wisconsin), a computer program capable of identifying likely signal sequences, was used to examine the N terminus of HG20. Several likely candidates for cleavage sites which would generate mature HG20 protein, *i.e.*, protein lacking the signal sequence, were identified. The results are shown in Figure 3.

25

## 30 EXAMPLE 2

### Expression of HG20 in normal and diseased adrenal tissue

Northern blots were performed to measure the amount of HG20 RNA in normal and diseased adrenal tissue. The results are shown in Table 2 below. The amount of the approximately 6.5 kb HG20 transcript is shown normalized to the amount of  $\beta$ -actin transcript.

35

Table 2

<u>Pathology</u>	<u>Profile</u>	<u>HG20</u> <u>RNA</u>	<u>Actin</u> <u>RNA</u>	<u>HG20</u> <u>/actin</u>
Pheochromocytoma	M, 30 yr	0.47	0.74	0.64
Adrenal carcinoma cortex	M, 69 yr	0.61	0.80	0.76
Adrenal adenoma cortex	M, 69 yr	0.62	1.15	0.54
Normal Adrenal	M, 26 yr	1.00	1.00	1.00

5                   The results shown in Table 2 indicate that HG20 expression is decreased in diseased states of the adrenal gland. Thus, increasing the concentration of HG20 in such diseased states is likely to be pharmacologically useful. Accordingly, one skilled in the art would expect agonists of HG20 to be pharmacologically useful.

10

## EXAMPLE 3

Tissue distribution of various HG20 RNA transcripts

Table 3, below, shows the results of experiments to measure the amount of HG20 RNA transcripts of various lengths in various  
15                   tissues. The results shown were derived from a multiple tissue Northern blot that was hybridized overnight in expressHyb solution (Clontech). Washing conditions were: 0.1X SSC, 0.1% SDS, at 60°C. A <sup>32</sup>P-random primer labelled Eco RI fragment containing the full-length native HG20 DNA was used as a hybridization probe. The greater  
20                   the number of plus signs in a particular tissue, the greater was the amount of HG20 RNA detected in that tissue.

Table 3

Tissue	6.5 kb	4.5 kb	4.0 kb	1.8 kb
cerebellum	++	+		
cerebral cortex	++++	+		
medulla	+	+		
occipital pole	+	+		
frontal lobe	+++	+		
temporal lobe	+++	+		
putamen	++	+		
spinal cord n=2	++	+		
amygdala	+++			
caudate nucleus	+	+		
corpus callosum	+	+		
hippocampus	++	+		
whole brain	+++	+		
substantia nigra	+	+		
subthalamic nucleus	+	+		
thalamus	++	+		
spleen		+		
thymus n=2		++		
prostate		++		
testis n=2	++	+	+++	
ovary		++	+	+
small intestine n=2		++		
colon (mucosal lining)		++		
peripheral blood leucocytes		++		
stomach n=2	+	+		
thyroid n=2	++	++++		
lymph node		+		
trachea		++		
adrenal gland	+++	+++	+	++++
bone marrow		++		
heart	+	++		
brain	+++++			
placenta		+		
lung		+		
liver		+		
skeletal muscle	+	++		
kidney		+		
pancreas	+	+		
adrenal medulla	+++			+
adrenal cortex	+++++		++	++

The distribution of HG20 RNA shown in Table 3 suggests that HG20 mediates activities of the central and peripheral nervous system.

5

## EXAMPLE 4

Distribution of HG20 mRNA in brain

Using in situ hybridisation, the distribution of HG20 mRNA in squirrel monkey brain was studied. Antisense oligonucleotide probes to HG20 were generated on an Applied Biosystems Model 394 DNA  
10 synthesiser and purified by preparative polyacrylamide electrophoresis. Probe 1: 5'ATC-TGG-GTT-TGT-TCT-CAG-GGT-GAT-GAG-CTT-CGG-CAC-GAA-TAC-CAG 3' (SEQ.ID.NO.:14); Probe2: 5' GCT-CTG-TGA-TCT-TCA-TTC-GCA-GGC-GAT-GGT-TTT-CTG-ACT-GTA-GGC 3' (SEQ.ID.NO.:15).  
15 Each oligonucleotide was 3'-end labelled with [<sup>35</sup>S] deoxyadenosine 5'-(thiotriphosphate) in a 30:1 molar ratio of <sup>35</sup>S-isotope:oligonucleotide using terminal deoxynucleotidyl transferase for 15 min at 37°C in the reaction buffer supplied (Boehringer). Radiolabelled oligonucleotide was separated from unincorporated nucleotides using Sephadex G50 spin  
20 columns. The specific activities of the labelled probes in several labelling reactions varied from 1.2-2.3 x 10<sup>9</sup> cpm/mg. Squirrel monkey brains were removed and fresh frozen in 1 cm blocks. 12 mm sections were taken and fixed for *in situ* hybridisation. Hybridisation of the sections was carried out according to the method of Sirinathsinghi et  
25 al., 1993, Neuroreports 4:175-178. Briefly, sections were removed from alcohol, air dried and 5 x10<sup>5</sup> cpm of each <sup>35</sup>S-labelled probe (both oligonucleotides) in 100 ml of hybridisation buffer was applied to each slide. Labelled "antisense" probe was also used in the presence of an excess (100x) concentration of unlabelled antisense probe to define non-  
30 specific hybridisation. Parafilm coverslips were placed over the sections which were incubated overnight (about 16 hr) at 37°C. Following hybridisation the sections were washed for 1 hr at 57°C in 1xSSC, then rinsed briefly in 0.1xSSC, dehydrated in a series of alcohols, air dried, and exposed to Amersham Hyperfilm bmax X-ray film.

Autoradiographs were analysed using a MCID computerised image analysis system (Image Research Inc., Ontario, Canada).

Highest levels of mRNA for HG20 were found in the hippocampus (dentate gyrus, CA3, CA2, and CA1). High levels were also seen in cortical regions (frontal, cingulate, temporal parietal, entorhinal, and visual) and the cerebellum, although medial septum, thalamic nuclei (medial-dorsal and lateral posterior), lateral geniculates, red nucleus, reticular formation, and griseum pontis all show expression of message. While there are many similarities with the distribution reported for the GABA<sub>B</sub> receptor mRNA in rat, one marked difference is that expression of HG20 mRNA in the monkey caudate and putamen is below the level of detection while cortical levels are high. In the rat, the GABA<sub>B</sub> receptor mRNA appears equally expressed in striatum as in cortex. Figure 4 illustrates these results.

#### EXAMPLE 5

##### Attempted recombinant expression of full-length HG20 protein

Following the cloning of HG20 DNA, attempts were made to express full-length HG20 protein (941 amino acids) using various eukaryotic cell lines and expression vectors. The cell lines that were used were: COS-7 cells, HEK293 cells, and frog melanophores. The expression vectors that were used to attempt to express the full-length HG20 protein were: pCR3.1 and pcDNA3.1 (Invitrogen, San Diego, CA) and pciNEO (promega)

All of the attempts to express full-length HG20 described above were unsuccessful. See, *e.g.*, Figure 7, second bar from the left, marked "HG20." See also Figure 5A, lane 1. Although the reason for these failures is not known, it is possible that the highly GC rich nature of the region of the HG20 mRNA that encodes amino acids 1-51 results in the formation of secondary structure in the mRNA that impedes translation. It was only after the construction of an expression vector that encodes a truncated HG20 protein, lacking the first 51 amino acids, that HG20 was successfully expressed. Figure 5A-B shows the results of the successful expression of an HG20 protein having amino acids 52-941. It is expected that expression of HG20 proteins having amino acids 53-

941, 54-941, 55-941, etc., could be accomplished in a similar manner. It is also expected that expression of HG20 proteins having the above-described amino termini but having different carboxyl termini could be accomplished in a similar manner as well. Thus, the expression of an  
5 HG20 protein having an amino terminus as listed above and having a truncated carboxyl terminus could be accomplished. Alternatively, the carboxyl terminus could be fused to non-HG20 amino acid sequences, forming a chimeric HG20 protein. It is also possible to express HG20  
10 HG20 sequences fused to the amino terminus.

Figure 5A shows the expression of amino acids 52-941 of HG20 as part of a chimeric or fusion protein with the FLAG epitope fused to the amino terminus of the HG20 sequences in a coupled *in vitro* transcription/translation experiment. Figure 5B shows the expression  
15 of amino acids 52-941 of HG20 as part of a chimeric or fusion protein with the FLAG epitope fused to the amino terminus of the HG20 sequences in COS-7 cells and melanophores. The expression vector used in this experiment was pcDNA3.1. The expression constructs used in  
20 Figure 5A-B also encoded a cleavable signal sequence from the influenza hemagglutinin gene that has been shown to facilitate the membrane insertion of G-protein coupled receptors (Guan et al., 1992, J. Biol. Chem. 267:21995-21998) and the fusion proteins were detected with anti-FLAG antibody. The expression constructs had also been engineered to  
25 contain a Kozak consensus sequence prior to the initiating ATG. The amino acid sequences of the hemagglutinin signal sequence and the FLAG epitope were:

[MKTIIALSYIFCLVFA] [DYKDDDDK] SEQ.ID.NO:17

HA signal peptide      FLAG epitope

30

Amino acids 57-941 have been expressed in mammalian cells as part of a chimeric protein. A chimeric construct of HG20 was made that consisted of bases -224 to 99 of the bovine GABA<sub>A</sub>  $\alpha$ 1 gene, a sequence encoding the c-myc epitope tag (amino acid residues 410-419 of the  
35 human oncogene product c-myc), a cloning site encoding the amino acid asparagine, and DNA encoding residues 57-941 of HG20. The resultant

chimeric protein has the amino acid sequence shown below, with the construct cloned into pcDNA1.1Amp (Invitrogen, San Diego, CA).

5    \_\_\_\_Bovine alpha 1 signal seq\_\_\_\_\_\_    \_\_c-myc\_\_    \_  
 MKKSPGLSDYLWAWTLFLSTLTGRSYGQPSLQD EQKLISEEDL N  
 \_res. 57-941 HG20\_  
 SIMGLMPLT... (SEQ.ID.NO.:18)

10    The three periods "..." indicate that the chimeric protein sequence extends until amino acid 941 of HG20.

15    The cell surface expression of this construct was verified using a cell surface ELISA technique. Briefly, HEK293 cells were seeded at  $\sim 1 \times 10^5$  cells per well in a 24 well tissue culture plate and allowed to adhere for 24 hours. Each well was transfected with a total of 1  $\mu$ g of DNA. In addition to tagged and un-tagged HG20 constructs, c-myc tagged GABA<sub>A</sub>  $\alpha$ 1 was transfected with GABA<sub>A</sub>  $\beta$ 1 as a positive control for cell surface expression. Two days after transfection, the cells were assayed for surface expression of the c-myc epitope using the 9E10 monoclonal antibody raised to the c-myc epitope, followed by HRP (horse  
 20    radish peroxidase) conjugated anti-mouse antibody (Promega) and colorimetric development using K-Blue (Bionostics). The results are shown in Figure 7. Figure 7 demonstrates that when HG20 is part of a chimeric protein, it can be expressed well in mammalian cells but that when attempts are made to express full-length HG20 (amino acids 1-941)  
 25    directly, *i.e.*, not as part of a chimeric protein, essentially no expression is observed.

## EXAMPLE 6

### Construction of Full Length Murine GABABR1a Coding Region

30    Using a combination of TFASTX (Pearson et al., 1997, Genomics 46:24-36) and TBLASTX (Altschul et al., 1997, Nucleic Acids Res. 25:3389-3402) searching programs against dbEST: Database of Expressed Sequence Tags (URL <http://www.ncbi.nlm.nih.gov/dbEST/index.html>), we identified partial cDNA clones in the EST collection  
 35    which encoded murine GABABR1a using the rat GABAB receptor

subunit cDNAs (GenBank Accession Numbers Y10369 and Y10370) as probe sequences (Kaupmann et al., 1997, Nature 386:239-246). Two of these ESTs (IMAGE Consortium clone identification numbers 472408 and 319196) were obtained (Research Genetics, Birmingham, Ala). The DNA sequences of both cDNA clones were determined using standard methods on an ABI 373a automated sequencer (Perkin-Elmer-Applied Biosystems, Foster City, CA).

The partial cDNAs were assembled by long accurate PCR using the following oligonucleotides: 472408 sense: 5' - GC GAATTC GGTACC ATG CTG CTG CTG CTG CTG GTG CCT - 3' (SEQ.ID.NO.:24), 472408 antisense: 5' - GG GAATTC TGG ATA TAA CGA GCG TGG GAG TTG TAG ATG TTA AA - 3' (SEQ.ID.NO.:25), 319196 sense: 5' - CCA GAATTC CCA GCC CAA CCT GAA CAA TC - 3' (SEQ.ID.NO.:26), 319196 antisense: 5' - CG GCGGCCGC TCA CTT GTA AAG CAA ATG TA - 3' (SEQ.ID.NO.:27) which amplified two fragments corresponding to the 5' 2,100 basepairs and 3' 1,000 basepairs of the murine GABABR1a coding region. The PCR conditions were 200 ng of cDNA template, 2.5 units of Takara LA Taq (PanVera, Madison, WI), 25 mM TAPS (pH 9.3), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 1 mM 2-mercaptoethanol, 100 mM each dNTP and 1 mM each primer with cycling as follows 94°C 1 min, 9 cycles of 98°C for 20 seconds, 72°C-56°C (decreases 2°C per cycle), 72°C for 30 seconds, followed by 30 cycles of 98°C for 20 seconds, 60°C for 3 minutes. A final extension at 72°C for 10 minutes was performed. PCR products were cloned into the TA-Cloning vector pCRII-TOPO (Invitrogen, San Diego, CA) following the manufacturers directions. Cloned PCR products were confirmed by DNA sequencing. To form full-length cDNA, the pCINeo mammalian expression vector was digested with EcoRI and NotI. The EcoRI fragment from PCR cloning of 472408 and the EcoRI/NotI product from PCR cloning of 319196 were ligated in a three part ligation with digested pCINeo vector. The resulting clones were screened by restriction digestion with SstI which cuts once in the vector and once in the 472408 derived fragment. The resulting expression clone is 2,903 basepairs in length. The overall cDNA length, including untranslated sequences, inferred from the full length of the two ESTs is 4,460 basepairs.



## EXAMPLE 7

Preparation of membrane fractions

P2 membrane fractions were prepared at 4°C as follows. Tissues or cells were washed twice with cold PBS, collected by centrifugation at 100xg for 7 min, and resuspended in 10 ml of buffer A: 5 mM Tris-HCl, 2 mM EDTA containing (1X) protease inhibitor cocktail Complete® tablets (Boehringer Mannheim), pH 7.4 at 4°C. Tissues or cells were disrupted by polytron homogenization, centrifuged at 100xg for 7 min to pellet unbroken cells and nuclei, and the supernatant collected. The resulting pellet was homogenized a second time in 10 ml of buffer A, centrifuged as described above and supernatant fractions saved. The pooled S1 supernatant was centrifuged at high speed (27 000xg for 20 min) and the pellet was washed once with buffer A, centrifuged (27 000xg for 20 min) and resuspended in buffer A to make the P2 membrane fraction, and stored at -80°C. Protein content was determined using the Bio-Rad Protein Assay Kit according to manufacturer instructions.

## EXAMPLE 8

20 Receptor filter-binding assays

Competition of [<sup>125</sup>I]CGP71872 binding experiments were performed with ~7 µg P2 membrane protein and increasing concentrations of cold ligand (10<sup>-12</sup>-10<sup>-3</sup> M). The concentration of radioligand used in the competition assays was 1 nM (final). Each concentration was examined in duplicate and incubated for 2 hours at 22°C in the dark in a total volume of 250 µL binding buffer: 50 mM Tris-HCl, 2.5 mM CaCl<sub>2</sub> (pH 7.4) with (1X) protease inhibitor cocktail Complete® tablets. Bound ligand was isolated by rapid filtration through a Brandel 96 well cell harvester using Whatman GF/B filters. Data were analysed by nonlinear least-squares regression using the computer-fitting program GraphPad Prism version 2.01 (San Diego).

## EXAMPLE 9

Photoaffinity labelling

P2 membranes were resuspended in binding buffer and incubated in the dark with 1 nM final concentration [<sup>125</sup>I]CGP71872 (2200 Ci/mmol) in a final volume of 1 ml for 2 h at 22°C. The membranes were centrifuged at 27,000xg for 10 min and the pellet was washed in ice-cold binding buffer, centrifuged at 27,000xg for 20 min, resuspended in 1 ml of ice-cold binding buffer, and exposed on ice 2 inches from 360 nm ultraviolet light for 10 min. Photolabelled membranes were washed, pelleted by centrifugation, and solubilized in sample buffer (50 mM Tris-HCl pH 6.5, 10% SDS, 10% glycerol, and 0.003% bromophenol blue with 10% 2-mercaptoethanol). Samples were electrophoresed on precast NOVEX 10% Tris-glycine gels, fixed, dried, and exposed to Kodak XAR film with an intensifying screen at -70°C.

15

## EXAMPLE 10

Immunoprecipitation and immunoblotting of GABA<sub>B</sub> receptors

Digitonin solubilized FLAG-tagged HG20 receptors were immunoprecipitated with a mouse anti-FLAG M2 antibody affinity resin (Kodak IBI) and immunoblot analysis conducted as previously described (Ng et al., 1996, Biochem. Biophys. Res. Comm. 227:200-204). Following washing of the immunoprecipitate, the pellet was resuspended in SDS sample buffer and subjected to SDS-PAGE and immunoblotted with affinity purified GABA<sub>B</sub>R1a-specific antibodies 1713.1 (raised against the peptide acetyl-DVNSRRDILPDYELKLC-amide (a portion of SEQ.ID.NO.:20)) and 1713.2 (raised against the peptide acetyl-CATLHNPTRVKLF EK-amide (a portion of SEQ.ID.NO.:20)).

25

## EXAMPLE 11

Melanophore functional assay

Growth of *Xenopus laevis* melanophores and fibroblasts was performed as described previously (Potenza et al., 1992, Anal. Biochem. 206:315-322). The cells (obtained from Dr. M.R. Lerner, Yale University) were collected by centrifugation at 200xg for 5 min at 4°C, and resuspended at  $5 \times 10^6$  cells per ml in ice cold 70% PBS, pH 7.0. DNA encoding the relevant GPCR was transiently transfected into melanophores by electroporation using a BTX ECM600 electroporator (Genetronics, Inc., San Diego, CA). To monitor the efficiency of transfection, two internal control GPCRs were used independently (pcDNA1amp-cannabinoid 2 and pcDNA3-thromboxane A2; (Lerner, 1994, Trends Neurosci. 17:142-146)). Cells were electroporated using the following settings: capacitance of 325 microfarad, voltage of 450 volts, and resistance of 720 ohms. Following electroporation, cells were mixed with fibroblast-conditioned growth medium and plated onto flat bottom 96 well microtiter plates (NUNC). 24 hrs after the transfection, the media was replaced with fresh fibroblast-conditioned growth media and incubated for an additional day at 27°C prior to assaying for receptor expression. For Gs/Gq-coupling responses (resulting in pigment dispersion), cells were incubated in 100 µl of 70% L-15 media containing 15 mM HEPES, pH 7.3, and melatonin (0.8 nM final concentration) for 1 hr in the dark at room temperature, and then incubated in the presence of melatonin (0.8 nM final concentration) for 1 h in the dark at room temperature to induce pigment aggregation. For Gi-coupled responses (resulting in pigment aggregation), cells were incubated in the presence of 100 µl/well of 70% L-15 media containing 2.5% fibroblast-conditioned growth medium, 2 mM glutamine, 100 µg /ml streptomycin, 100 units/ml penicillin and 15 mM HEPES, pH 7.3, for 30 min in the dark at room temperature to induce pigment dispersion. Absorbance readings at 600 nm were measured using a Bio-Tek Elx800 Microplate reader (ESBE Scientific) before (Ai) and after (Af) incubation with ligand (GABA; 1.5 hr in the dark at room temperature).

## EXAMPLE 12

Stable and transient transfections and determination of cAMP response in HEK293 cells

5 HG20 and murine GABABR1a cDNAs were subcloned into pcDNA3.1 (Invitrogen, San Diego, CA) and used to transfect HEK293 cells. Stably expressing cells were identified after selection in geneticin (0.375 mg/ml) by dot blot analysis. For co-expression experiments, the stable cell lines hgb2-42 (expressing HG20) and rgl1a-50 (expressing murine GABABR1a) were transiently transfected with murine  
10 GABABR1a and HG20, respectively, in pcDNA3.1 and cells were assayed for cAMP responses.

Wild-type HEK293 cells, or HEK293 cells stably and transiently expressing HG20 and murine GABABR1a receptors were lifted in 1X PBS, 2.5 mM EDTA, counted, pelleted and resuspended at 1.5  
15 x 10<sup>5</sup> cells per 100 µl in Krebs-Ringer-Hepes medium (Blakely et al., 1991, Anal. Biochem. 194:302-308), 100 mM Ro 20-1724 (RBI) and incubated at 37°C for 20 min. 100 µl of cells was added to 100 µl of prewarmed (37°C, 10 min) Krebs-Ringer-Hepes medium, 100 mM Ro 20-1724 without or with agonist and/or 10 µM forskolin. Incubations with GABA included  
20 100 µM aminooxyacetic acid (a GABA transaminase inhibitor) to prevent breakdown of GABA and 100 µM nipecotic acid to block GABA uptake. Following a 20 min incubation at 37°C, the assay was terminated by setting the cells on ice and centrifuging at 2,000 rpm for 5 min at 4°C. 175 ml of assay solution was removed and replaced with 175 ml of 0.1 N  
25 hydrochloric acid, 0.1 mM calcium chloride and cells were set on ice for 30 min and then stored at -20°C. cAMP determinations were made using a solid phase modification (Maidment et al., 1989, Neurosci. 33:549-557) of the cAMP radioimmunoassay described by Brooker et al. 1979, Adv. Cyclic Nucl. Res. 10:1-33) and previously reported in Clark et al., 1998, Mol. Endocrinol. 12:193-206). Immulon II removable wells  
30 (Dynatech; Chantilly, VA) were coated overnight with 100 µl of protein G (1mg/ml in 0.1M NaHCO<sub>3</sub>, pH 9.0) at 4°C. Prior to use, protein G-coated plates were rinsed with PBS-gelatin-Tween (phosphate buffered saline containing 0.1% gelatin, 0.2% Tween-20) 3 times quickly, and then once  
35 for 30 minutes. Following the rinse with PBS-gelatin-Tween, the RIA

was set up by adding 100 µl 50 mM sodium acetate, pH 4.75, cAMP standards or aliquots from treated cells, 5,000-7,000 cpm <sup>125</sup>I-succinyl cAMP, and 25 µl of a sheep antibody to cAMP diluted in 50 mM sodium acetate, pH 4.75 (Atto instruments; dilution of stock to 2.5x10<sup>-5</sup>,  
 5 determined empirically) to the plates in a final volume of 175 µl. Plates were incubated 2 hr at 37°C or overnight at 4°C, rinsed 3 times with sodium acetate buffer, blotted dry, and then individual wells were broken off and bound radioactivity was determined in a gamma counter.

10

## EXAMPLE 13

*In situ* hybridization for co-localization experiments

Preparation of rat brain sections, prehybridization and hybridization of rat brain slices was performed as described previously (Bradley et al., 1992, J. Neurosci. 12:2288-2302;  
 15 <http://intramural.nimh.nih.gov/lcmr/snge/Protocol.html>). Adjacent coronal rat brain sections were hybridized with labeled antisense and sense riboprobes directed against HG20 (GenBank accession number AF058795) or murine GABABR1a.

HG20 probes were generated by amplification of HG20 with  
 20 JC216 (T3 promotor/primer and bases 1172-1191) paired with JC217 (T7-promotor/primer and bases 1609-1626) or with JC218 (T3 promotor/primer and bases 2386-2405) paired with JC219 (T7 promotor/primer and bases 2776-2793):

(JC216: cgcgcaattaaccctcactaaaggACAACAGCAAACGTTTCAGGC  
 25 (SEQ.ID.NO.:28);

JC217: gcgcgtaatacg actcactatagggCATGCCTATGATGGTGAG  
 (SEQ.ID.NO.:29);

JC218: cgcgcaattaaccctcactaaagg CTGAGGACAAACCCTGACGC  
 (SEQ.ID.NO.:30);

30 JC219: gcgcgtaatacgactcactatagggGATGTC TTCTATGGGGTC;  
 (SEQ.ID.NO.:31)).

Murine GABABR1a probes were generated by amplification of murine GABABR1a with JC160 (T3 promotor/primer and bases 631-648) paired with JC161 (T7 promotor/primer and bases 1024-1041):

(JC160: cgcgcaattaaccctcactaaaggAAGCTTATCCACCACGAC

(SEQ.ID.NO.:32);

JC161:gcgcgtaa tacgactcactatagggAGCTGGATCCGAGAAGAA

(SEQ.ID.NO.:33)).

- 5 For colocalization experiments, murine GABABR1a probes were labeled with digoxigenin-UTP and detected using a peroxidase-conjugated antibody to digoxigenin and TSA amplification involving biotinyl tyramide and subsequent detection with streptavidin-conjugated fluorescein. HG20 probes were radiolabelled
- 10 (<http://intramural.nimh.nih.gov/lcmr/snge/Protocol.html>). For individual hybridizations, murine GABABR1a and HG20 riboprobes were radiolabeled with <sup>35</sup>S-UTP and detected as described previously (Bradley et al., 1992, J. Neurosci. 12:2288-2302; <http://intramural.nimh.nih.gov/lcmr/snge/Protocol.html>). Brain slices
- 15 were either hybridized with individual radiolabelled probes or, for colocalization studies, simultaneously with probes to both murine GABABR1a and HG20 receptors. Detection of the radiolabeled HG20 probe was performed after detection of the digoxigenin-labeled rgl1 probe on the same brain slices.

20

#### EXAMPLE 14

##### Construction of N-terminal and C-terminal fragments of murine GABABR1a

- The N-terminal fragment of murine GABABR1a,
- 25 comprising amino acid positions 1-625, was generated by PCR. The coding sequence of the N-terminal fragment was amplified by using primer pairs: NFP-CJ7843F139 (5'- ACC ACT GCT AGC ACC GCC ATG CTG CTG CTG CTG CTT CTG C -3'; SEQ.IS.NO.:34) and NRP-CJ7844 (3'- GG GTG CGA GCA ATA TAG GTC TTA AGG GTC GGC CGC
- 30 CGG CGT CAC CA -5'; ; SEQ.IS.NO.:35). Similarly, the C-terminal fragment, amino acid positions 588-942, was generated by PCR using primer pairs: CFP-CJ7845 (5'- ACC ACT GCT AGC ACC GCC ATG CAG AAA CTC TTT ATC TCC GTC TCA GTT CTC TCC AGC-3'; ; SEQ.IS.NO.:36) and CRP-CJ7846 (3'- CAG CTC ATG TAA ACG AAA
- 35 TGT TCA CTC GCC GGC CGC CGG CGT CAC CA-5'; ; SEQ.IS.NO.:37).

PCR reactions were carried out using the Advantage-HF PCR kit (Clontech, Paolo Alto, CA) with 0.2 ng of murine GABABR1a DNA as the template, and 10  $\mu$ M of each primer according to manufacturer instructions. The PCR conditions were as follows: precycle  
5 denaturation at 94°C for 1 min, and then 35 cycles at 94°C (15 s), annealing and extension at 72°C (3 min), followed by a final extension for 3 min at 72°C. The PCR products, N-gb 1a and C-gb 1a DNA, flanked by Nhe1 and Not1 sites, were digested and subcloned into the Nhe1/Not1 site of pcDNA3.1 (Invitrogen, San Diego, Ca).

10

## EXAMPLE 15

Cell culture and preparation of membrane fractions for binding experiments using N-terminal and C-terminal GABABR1a fragments

COS-7 cells (ATCC) were cultured in DMEM, 10% bovine  
15 serum, 25 mM HEPES, and antibiotics and transiently transfected with murine gb1a/pcDNA3.1 (encoding full-length GABABR1a), N-gb 1a/pcDNA3.1 (encoding the N-terminal fragment of GABABR1a; see Example 14) or C-gb 1a/pcDNA3.1 (encoding the C-terminal fragment of GABABR1a; see Example 14) using Lipofectamine reagent (Gibco BRL)  
20 following the conditions recommended by the manufacturer. At 48 h post-transfection, P2 membrane fractions were prepared at 4°C as follows: Cells were washed twice with cold PBS, collected by centrifugation at 100xg for 7 min, and resuspended in 10 ml of buffer A: 5 mM Tris-HCl, 2 mM EDTA containing (1X) protease inhibitor cocktail  
25 Complete® tablets (Boehringer Mannheim), pH 7.4 at 4°C. Cells were disrupted by polytron homogenization, centrifuged at 100xg for 7 min to pellet unbroken cells and nuclei, and the supernatant collected. The resulting pellet was homogenized a second time in 10 ml of buffer A, centrifuged as described above and supernatant fractions saved. The  
30 pooled S1 supernatant was centrifuged at high speed (27,000xg for 20 min) and the pellet was washed once with buffer A, centrifuged (27,000xg for 20 min), resuspended in buffer A to make the P2 membrane fraction, and stored at -80°C. Protein content was determined using the Bio-Rad Protein Assay Kit according to manufacturer instructions.

## EXAMPLE 16

In vitro transcription/translation of GABABR1a and N-terminal and C-terminal fragments

- 5        *In vitro* transcription coupled translation reactions were performed in the presence of [<sup>35</sup>S]-methionine in the TNT Coupled Reticulocyte Lysate system (Promega, WI) using the pcDNA3.1 plasmid containing the full-length GABABR1a, N-gb1a, and C-gb1a DNAs. Translation products were analysed by electrophoresis on 8-16% Tris-  
10   Glycine gradient gels (Novex pre-cast gel system) under denaturing and reducing conditions. Gels were fixed, treated with enlightening fluid (NEN), dried and exposed to Kodak X-AR film at -70°C for 4 to 24 h. Analysis of the results of these *in vitro* transcription coupled translation reactions confirmed that the constructs whose production is described in  
15   Example 14 directed the expression of the appropriate GABABR1a fragments (see Figure 17A).

## EXAMPLE 17

Immunoblot analysis for experiments with N-terminal and C-terminal fragments of GABABR1a

- 20        The expression of full-length and N-terminal and C-terminal GABABR1a fragments *in vivo* was confirmed by immunoblot analysis. Membranes were solubilized in SDS sample buffer consisting of 50 mM Tris-HCl pH 6.5, 10% SDS, 10% glycerol, and 0.003%  
25   bromophenol blue with 10% 2-mercaptoethanol and separated on SDS-PAGE. The full-length receptor and N-terminal fragment were detected using affinity purified rabbit GABABR1a polyclonal antibody 1713.1 (acetyl-DVNSRRDILPDYELKLC-amide; a portion of SEQ.ID.NO.:20) and 1713.2 (acetyl-CATLHNPTRVKLFEEK-amide; a portion of  
30   SEQ.ID.NO.:20) (Quality Control Biochemicals (Hopkinton, MA). The C-terminal fragment was detected using a GABABR1a antibody raised against the C-terminal tail of the receptor (acetyl-PSEPPDRLSCDGSRVHLLYK-amide; SEQ.ID.NO.:20) (Chemicon Int.,



Inc., Canada).

### EXAMPLE 18

5     Receptor filter-binding assays for experiments with N-terminal and C-terminal fragments of GABABR1a

Competition of [<sup>125</sup>I] CGP71872 binding experiments were performed with ~7 µg P2 membrane protein and increasing concentrations of cold ligand (10<sup>-12</sup>-10<sup>-3</sup> M). The concentration of radioligand used in the competition assays was 1 nM (final). Each  
10     concentration was examined in duplicate and incubated for 2 hr at 22°C in the dark in a total volume of 250 µL binding buffer: 50 mM Tris-HCl, 2.5 mM CaCl<sub>2</sub> (pH 7.4) with (1X) protease inhibitor cocktail Complete<sup>®</sup> tablets. Bound ligand was isolated by rapid filtration through a Brandel 96 well cell harvester using Whatman GF/B filters. Data were analysed  
15     by nonlinear least-squares regression using the computer-fitting program GraphPad Prism version 2.01 (San Diego).

### EXAMPLE 19

20     Photoaffinity labeling for experiments with N-terminal and C-terminal fragments of GABABR1a

P2 membranes were resuspended in binding buffer, and incubated in the dark with 1 nM final concentration [<sup>125</sup>I]CGP71872 (2200 Ci/mmol) in a final volume of 1 ml for 2 h at 22°C. The membranes were centrifuged at 27,000xg for 10 min and the pellet was washed in  
25     ice-cold binding buffer, centrifuged at 27,000xg for 20 min and resuspended in 1 ml of ice-cold binding buffer and exposed on ice 2 inches from 360 nm ultraviolet light for 10 min. Photolabeled membranes were washed and membranes pelleted by centrifugation and solubilized in sample buffer (50 mM Tris-HCl pH 6.5, 10% SDS, 10%  
30     glycerol, and 0.003% bromophenol blue with 10% 2-mercaptoethanol). Samples were electrophoresed on precast NOVEX 10% Tris-glycine gels,

fixed, dried, and exposed to Kodak XAR film with an intensifying screen at -70°C.

## EXAMPLE 20

5

### Construction of the FLAG epitope-tagged HG20 and detection of expression in vitro and in COS-1 cells

The FLAG epitope-tagged HG20 receptor subunit was  
10 constructed by PCR using a sense primer encoding a modified influenza hemagglutinin signal sequence (MKTIALSYIFCLVFA; a portion of SEQ.ID.NO.:17) (Jou et al., 1980, Cell 19:683-696) followed by an antigenic FLAG epitope (DYKDDDDK; a portion of SEQ.ID.NO.:17) and DNA  
15 encoding amino acids 52-63 of HG20 and an antisense primer encoding amino acids 930-941 of the HG20 in a high-fidelity PCR reaction with HG20/pCR 3.1 as a template. HG20/pCR 3.1 is a plasmid that contains full-length HG20 (SEQ.ID.NO.:2) cloned into pCR3.1. The nucleotide  
20 sequences of the sense and antisense primers are: sense: 5'-GCC GCT AGC GCC ACC ATG AAG ACG ATC ATC GCC CTG AGC TAC ATC TTC TGC CTG GTA TTC GCC GAC TAC AAG GAC GAT GAT GAC  
AAG AGC AGC CCG CCG CTC TCC ATC ATG GGC CTC ATG CCG CTC-3', (SEQ.ID.NO.:38); antisense: 5'-GCC TCT AGA TTA CAG GCC CGA GAC CAT GAC TCG GAA GGA GGG TGG CAC-3'.  
(SEQ.ID.NO.:39). The PCR conditions were: precycle denaturation at  
25 94°C for 1 min, 94°C for 30 sec, annealing and extension at 72°C for 4 min for 25 cycles, followed by a 7 min extension at 72°C. The PCR product, SF-HG20 DNA, flanked by NheI and XbaI sites was subcloned into the NheI/XbaI site of pcDNA3.1 (Invitrogen, San Diego, Ca) to give rise to the expression construct SF-HG20/pcDNA3.1. The sequence of  
30 this construct was verified on both strands.

The SF-HG20 receptor was expressed in an *in vitro* coupled transcription/translation reaction using the TNT Coupled Reticulocyte Lysate system (Promega, WI) in the presence of [<sup>35</sup>S]methionine  
35 according to the manufacturer instructions. Radiolabeled proteins were analyzed by electrophoresis on 8-16% Tris-Glycine gradient gels (Novex pre-cast gel system) under denaturing and reducing conditions.

Gels were fixed and treated with Enlightening fluid (NEN), dried and exposed to Kodak X-AR film at -70°C.

COS-1 cells (ATCC, CRL 1650) were cultured in DMEM, 10% bovine serum, 25 mM HEPES, pH 7.4, and 10 units/mL penicillin- 10  
5 µg/mL streptomycin. Transient transfection of COS-1 cells with SF-  
HG20/pcDNA 3.1 was carried out using Lipofectamine reagent (Gibco BRL) following the conditions recommended by the manufacturer. At 48  
h post-transfection, crude membranes were prepared and receptors  
were solubilized with digitonin and immunoprecipitated with anti-  
10 FLAG M2 affinity gel resin (IBI) under previously described conditions  
(Ng et al., 1993). The immunoprecipitate was washed and solubilized in  
SDS sample buffer, sonicated, electrophoresed, and blotted on to  
nitrocellulose membrane as described (Ng et al., 1993). The FLAG-  
tagged HG20 receptor was detected using an anti-FLAG antibody (Santa  
15 Cruz Biotech., Inc.) by following a chemilumescence protocol of the  
manufacturer (NEN).

## EXAMPLE 21

### Kir channel activity in *Xenopus* oocytes

20 With the following modifications, *Xenopus* oocytes were  
isolated as described (Hébert et al., 1994, Proc. R. Soc. Lond. B 256:253-  
261) from live frogs supplied by Boreal, Inc. After a brief (10 min)  
hypertonic shock with 125 mM potassium phosphate pH 6.5, oocytes  
were allowed to recover in Barth's solution for 1-2 hr. cDNA constructs  
25 for human Kir 3.1, Kir 3.2 channel isoforms (generous gifts from Dr.  
Hubert Van Tol, University of Toronto), and  $G\alpha 1$  (a generous gift of Dr.  
Maureen Linder, Washington University) were linearized by restriction  
enzymes and purified using GeneClean (Bio 101). Murine GABABR1a or  
FLAG-HG20 clones were subcloned into pT7TS (a generous gift of Dr.  
30 Paul Krieg, University of Texas) before linearization and transcription.  
Capped cRNA was made using T7 RNA polymerase and the mMessage  
mMachine (Ambion). Individual oocytes were injected with 5-10 ng (in  
25-50 nL) of Kir3.1 and Kir3.2 constructs with mRNAs for murine  
GABABR1a or FLAG-HG20 and in combination with  $G\alpha 1$  as well. Kir  
35 currents were also evaluated in oocytes co-injected with Kir3.1, Kir3.2,

murine GABA<sub>B</sub>R1a and FLAG-HG20 mRNAs. Currents were recorded after 48 hr. Standard recording solution was KD-98, 98 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM K-HEPES, pH 7.5, unless otherwise stated.

Microelectrodes were filled with 3 M KCl and had resistances of 1-3 MW and 0.1-0.5 MW for voltage and current electrodes, respectively. In addition, current electrodes were backfilled with 1% agarose (in 3M KCl) to prevent leakage as described (Hébert et al., 1994, Proc. R. Soc. Lond. B 256:253-261). Recordings were made at room temperature using a Geneclamp 500 amplifier (Axon Instruments). Oocytes were voltage clamped and perfused continuously with different recording solutions. Currents were evoked by 500 msec voltage commands from a holding potential of -10 mV, delivered in 20 mV increments from -140 to 60 mV to test for inward rectifying potassium currents. Data were recorded at a holding potential of -80 mV and drugs were added to the bath with a fast perfusion system. Data collection and analysis were performed using pCLAMP v6.0 (Axon Instruments) and Origin v4.0 (MicroCal) software. For subtraction of endogenous and leak currents, records were obtained in ND-96, 96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM Na-HEPES and these were subtracted from recordings in KD-98 before further analysis.

## EXAMPLE 22

### Radiation Hybrid mapping of HG20

Radiation hybrid analysis assigned the HG20 gene to chromosome 9, placing it 4.81 cR from the WI-8684 marker on the GeneBridge 4 panel of 93 RH clones of the whole human genome. Searching of the OMIM database with D9S176 and D9S287 markers proximal to the HG20 gene revealed it to map proximal to the hereditary sensory neuropathy type 1 (HSN-1) locus, a ~8 cM region flanked by D9S176 and 9S318 (Nicholson et al., 1996, Nature Genetics 13, 101-104) (Figure 20). HSN-1 is the most common form of a group of degenerative disorders of sensory neurons characterized by a progressive degeneration of dorsal root ganglion and motor neurons that lead to distal sensory loss, distal muscle wasting and weakness, and neural deafness, among a number of other neuronally related deficits

(Nicholson et al., 1996, Nature Genetics 13, 101-104). FCMD (Fukuyama congenital muscular dystrophy) and DYS (dysautonomia, another type of HSN) also map to this area. Candidate gene(s) in these disorders are likely critical to the development, survival, and differentiation of  
5 neurons.

A human BAC library was screened using the EcoRI fragment containing the full-length HG20 DNA, and end-sequencing was performed on BAC clones designated 6D18, 168K19, 486B24, and 764N4. The primer pair: ngf1t7+ (5'-AAC AGT CAA AAC CCA CCC  
10 AG-3'; SEQ.ID.NO.:40) and ngf1t7- (5'-AAC AGT TTC CAG CTG TGC CT-3'; SEQ.ID.NO.:41) were identified for radiation hybrid mapping of the HG20 gene on the GENEBRIDGE 4 panel. BAC library screening and radiation hybrid mapping were performed by Research Genetics (Huntsville, AL).

15  
The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such  
20 modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

## WHAT IS CLAIMED:

1. An isolated DNA molecule encoding an HG20 polypeptide comprising an amino acid sequence selected from the group consisting of:  
5       SEQ.ID.NO.:2;  
          Positions 9-941 of SEQ.ID.NO.:2;  
          Positions 35-941 of SEQ.ID.NO.:2;  
          Positions 36-941 of SEQ.ID.NO.:2;  
10       Positions 38-941 of SEQ.ID.NO.:2;  
          Positions 39-941 of SEQ.ID.NO.:2;  
          Positions 42-941 of SEQ.ID.NO.:2;  
          Positions 44-941 of SEQ.ID.NO.:2;  
          Positions 46-941 of SEQ.ID.NO.:2;  
15       Positions 52-941 of SEQ.ID.NO.:2; and  
          Positions 57-941 of SEQ.ID.NO.:2.
2. The isolated DNA molecule of claim 1 comprising a nucleotide sequence selected from the group consisting of:  
20       SEQ.ID.NO.:1;  
          Positions 293-3,115 of SEQ.ID.NO.:1;  
          Positions 317-3,115 of SEQ.ID.NO.:1;  
          Positions 395-3,115 of SEQ.ID.NO.:1;  
          Positions 398-3,115 of SEQ.ID.NO.:1;  
25       Positions 404-3,115 of SEQ.ID.NO.:1;  
          Positions 407-3,115 of SEQ.ID.NO.:1;  
          Positions 416-3,115 of SEQ.ID.NO.:1;  
          Positions 422-3,115 of SEQ.ID.NO.:1;  
          Positions 428-3,115 of SEQ.ID.NO.:1;  
30       Positions 446-3,115 of SEQ.ID.NO.:1; and  
          Positions 461-3,115 of SEQ.ID.NO.:1.
3. An isolated DNA molecule that hybridizes under stringent conditions to the DNA molecule of claim 2.  
35
4. An expression vector comprising the DNA of claim 1.

5. A recombinant host cell comprising the expression vector of claim 4.

5 6. The recombinant cell of claim 5 further comprising an expression vector comprising DNA encoding a protein selected from the group consisting of:

10 7. A protein, substantially free from other proteins, comprising an HG20 protein having an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;

Positions 9-941 of SEQ.ID.NO.:2;

15 Positions 35-941 of SEQ.ID.NO.:2;

Positions 36-941 of SEQ.ID.NO.:2;

Positions 38-941 of SEQ.ID.NO.:2;

Positions 39-941 of SEQ.ID.NO.:2;

Positions 42-941 of SEQ.ID.NO.:2;

20 Positions 44-941 of SEQ.ID.NO.:2;

Positions 46-941 of SEQ.ID.NO.:2;

Positions 52-941 of SEQ.ID.NO.:2; and

Positions 57-941 of SEQ.ID.NO.:2.

25 8. A heterodimer comprising the protein of claim 7 and a G-protein coupled receptor protein where the heterodimer is substantially free from other proteins.

30 9. The heterodimer of claim 8 where the heterodimer is held together by N-terminal Sushi repeats, C-terminal alpha-helical interacting domains, coiled-coil domains, transmembrane interactions, or disulfide bonds.

35 10. A polypeptide comprising a coiled-coil domain from a first GABAB receptor subunit and no other contiguous amino acid sequences longer than 5 amino acids from the first GABAB receptor subunit where the coiled-coil domain is present in the C-terminus of the

GABA<sub>B</sub> receptor subunit and mediates heterodimerization of the first GABA<sub>B</sub> receptor subunit with a second GABA<sub>B</sub> receptor subunit.

11. The polypeptide of claim 10 where the coiled-coil  
5 domain is selected from the group consisting of: positions 756-829 of SEQ.ID.NO.:2; positions 779-814 of SEQ.ID.NO.:2; positions 886-949 of SEQ.ID.NO.:21; and positions 889-934 of SEQ.ID.NO.:21.

12. An isolated DNA molecule encoding a GABA<sub>B</sub>R1a  
10 polypeptide comprising the amino acid sequence SEQ.ID.NO.:20.

13. A protein, substantially free from other proteins,  
comprising a GABA<sub>B</sub>R1a protein having the amino acid sequence  
SEQ.ID.NO.:20.  
15

14. A method for determining whether a substance binds  
GABA<sub>B</sub> receptors and is thus a potential agonist or antagonist of the  
GABA<sub>B</sub> receptor that comprises:

(a) providing cells comprising an expression vector  
20 encoding HG20 and an expression vector encoding GABA<sub>B</sub>R1a or  
GABA<sub>B</sub>R1b;

(b) culturing the cells under conditions such that HG20  
and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b are expressed and heterodimers of HG20  
and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b are formed;

25 (c) exposing the cells to a labeled ligand of GABA<sub>B</sub>  
receptors in the presence and in the absence of the substance;

(d) measuring the binding of the labeled ligand to the  
heterodimers of HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b;

30 where if the amount of binding of the labeled ligand is less  
in the presence of the substance than in the absence of the substance,  
then the substance is a potential agonist or antagonist of GABA<sub>B</sub>  
receptors.

15. A method of identifying agonists and antagonists of  
35 HG20 comprising:

(a) providing test cells by transfecting cells with:



- (1) an expression vector that directs the expression of HG20 in the cells; and
- (2) an expression vector that directs the expression of GABABR1a or GABABR1b in the cells;
- 5 (b) exposing the test cells to a substance that is suspected of being an agonist of the GABAB receptor;
- (c) measuring the amount of a functional response of the test cells that have been exposed to the substance;
- (d) comparing the amount of the functional response
- 10 exhibited by the test cells with the amount of the functional response exhibited by control cells;
- wherein if the amount of the functional response exhibited by the test cells differs from the amount of the functional response exhibited by the control cells, the substance is an agonist or antagonist of
- 15 the GABAB receptor;
- where the control cells are cells that have not been transfected with HG20 and GABABR1a or GABABR1b but have been exposed to the substance or are test cells that have not been exposed to the substance.
- 20
16. A method of producing functional GABAB receptors in cells comprising:
- (a) transfecting cells with:
- (1) an expression vector that directs the
- 25 expression of HG20 in the cells; and
- (2) an expression vector that directs the expression of GABABR1a or GABABR1b in the cells;
- (b) culturing the cells under conditions such that heterodimers of HG20 and GABABR1a or GABABR1b are formed where
- 30 the heterodimers constitute functional GABAB receptors.
17. An antibody that binds specifically to HG20 where HG20 has an amino acid sequence selected from the group consisting of:
- SEQ.ID.NO.:2;
- 35 Positions 9-941 of SEQ.ID.NO.:2;
- Positions 35-941 of SEQ.ID.NO.:2;
- Positions 36-941 of SEQ.ID.NO.:2;

5 Positions 38-941 of SEQ.ID.NO.:2;  
Positions 39-941 of SEQ.ID.NO.:2;  
Positions 42-941 of SEQ.ID.NO.:2;  
Positions 44-941 of SEQ.ID.NO.:2;  
Positions 46-941 of SEQ.ID.NO.:2;  
Positions 52-941 of SEQ.ID.NO.:2; and  
Positions 57-941 of SEQ.ID.NO.:2.

10 18. A method of expressing a truncated version of HG20 protein comprising:

- (a) transfecting a host cell with a expression vector that encodes an HG20 protein that has been truncated at the amino terminus;
- 15 (b) culturing the transfected cells of step (a) under conditions such that the truncated HG20 protein is expressed.

19. A chimeric HG20 protein having an amino acid sequence of HG20 selected from the group consisting of:

20 Positions 51-941 of SEQ.ID.NO.:2;  
Positions 52-941 of SEQ.ID.NO.:2;  
Positions 53-941 of SEQ.ID.NO.:2;  
Positions 54-941 of SEQ.ID.NO.:2;  
Positions 55-941 of SEQ.ID.NO.:2;  
Positions 56-941 of SEQ.ID.NO.:2;  
25 Positions 57-941 of SEQ.ID.NO.:2; and  
Positions 58-941 of SEQ.ID.NO.:2;  
covalently linked at the N-terminus with a non-HG20 amino acid sequence.

1/32  
FIGURE 1A

1 CCGCCCTCCC CCGGCCGAGC TCCAGGGCTG CCGCCTAGCA GCTCCCGGCG  
51 GGAGAGCGGT TCAGAGCTCG CTCCACCCCC TTCCCGGCGT GATTGATCCG  
101 TCACGGGCGC CTCCGCTGCC GCCGCCGCCG CCGCGGCCGT TCTGAGCCGA  
151 GCCGGAACCC TAGCCCGAGA CGGAGCCGGG GCCCGGGCCG GCGCCATTGC  
201 GCGGGCGCCG CGGGAAGACC TTGGCGCGGG GCGGCGGGCC GGGCCAGGCC  
251 ATGCGGGCCG AGTGAGCCGG CGCCCGCAGC CCGCGGCGCG GCATGGCTTC  
301 CCCGCGGAGC TCCGGGCAGC CCGGGCCGCC GCCGCCGCCG CCACCGCCGC  
351 CCGCGCGCCT GCTACTGCTA CTGCTGCTGC CGCTGCTGCT GCCTCTGGCG  
401 CCCGGGGCCT GGGGCTGGGC GCGGGGCGCC CCCCGGCCGC CGCCCAGCAG  
451 CCCGCCGCTC TCCATCATGG GCCTCATGCC GTCACCAAG GAGGTGGCCA  
501 AGGGCAGCAT CGGGCGCGGT GTGCTCCCCG CCGTGGAAT GGCCATCGAG  
551 CAGATCCGA ACGATCACT CCTGCGCCCC TACTTCCTCG ACCTCGGCT  
601 CTATGACACG GAGTGCACG ACGCAAAAGG GTTGAAAGCC TTCTACGATG  
651 CAATAAAATA CGGGCCGAAC CACTTGATGG TGTITGGAGG CGTCTGTCCA  
701 TCCGTACAT CCATCATTGC AGAGTCCCTC CAAGGCTGGA ATCTGGTGCA  
751 GCTTCTTTT GCTGCAACCA CGCCTGTTCT AGCCGATAAG AAAAAATACC  
801 CTTATTTCTT TCGGACCGTC CCATCAGACA ATGCGGTGAA TCCAGCCATT  
851 CTGAAGTTGC TCAAGCACTA CCAGTGGAAG CGCGTGGGCA CGCTGACGCA  
901 AGACGTTAG AGGTTCTCTG AGGTGCGGAA TGACCTGACT GGAGTTCTGT  
951 ATGGCGAGGA CATTGAGATT TCAGACACCG AGAGCTTCTC CAACGATCCC  
1001 TGTACCAGTG TCAAAAAGCT GAAGGGGAAT GATGTGCGGA TCATCCTTGG  
1051 CCAGTTTGAC CAGAATATGG CAGCAAAAGT GTTCTGTTGT GCATACGAGG  
1101 AGAACATGTA TGGTAGTAAA TATCAGTGGA TCATCCGGG CTGGTACGAG  
1151 CCTTCTTGGT GGGAGCAGGT GCACACGGAA GCCAACTCAT CCCGCTGCCT  
1201 CCGGAAGAAT CTGCTTGCTG CCATGGAGGG CTACATTGGC GTGGATTTCG  
1251 AGCCCCTGAG CTCCAAGCAG ATCAAGACCA TCTCAGGAAA GACTCCACAG  
1301 CAGTATGAGA GAGAGTACAA CAACAAGCGG TCAGGCGTGG GCGCCAGAA  
1351 GTTCCACGGG TACGCCTACG ATGGCATCTG GGTCATCGCC AAGACACTGC  
1401 AGAGGGCCAT GGAGACACTG CATGCCAGCA GCCGGCACCA GCGGATCCAG  
1451 GACTTCAACT ACACGGACCA CACGCTGGG AGGATCATCC TCAATGCCAT  
1501 GAACGAGACC AACTTCTTCG GGGTCACGGG TCAAGTTGTA TTCCGGAATG  
1551 GGGAGAGAAT GGGGACCATT AAATTTACTC AATTTCAAGA CAGCAGGGAG  
1601 GTGAAGGTGG GAGAGTACAA CGCTGTGGCC GACACACTGG AGATCATCAA  
1651 TGACACCATC AGGTTCCAAG GATCCGAACC ACCAAAAGAC AAGACCATCA  
1701 TCCTGGAGCA GCTGCGGAAG ATCTCCCTAC CTCTCTACAG CATCCTCTCT  
1751 GCCCTACCA TCCTCGGGAT GATCATGGCC AGTGCTTTTC TCTTCTTCAA  
1801 CATCAAGAAC CGGAATCAGA AGCTCATAAA GATGTCGAGT CCATACATGA  
1851 ACAACCTTAT CATCCTTGGA GGGATGCTCT CCTATGCTTC CATATTTCTC  
1901 TTTGGCCTTG ATGGATCCTT TGTCTCTGAA AAGACCTTTG AAACACTTTG  
1951 CACCGTCAGG ACCTGGATTG TCACCGTGGG CTACACGACC GCTTTTGGGG  
2001 CCATGTTTGC AAAGACCTGG AGAGTCCACG CCATCTTCAA AAATGTGAAA  
2051 ATGAAGAAGA AGATCATCAA GGACCAGAAA CTGCTTGTA TCGTGGGGGG  
2101 CATGCTGCTG ATCGACCTGT GTATCCTGAT CTGCTGGCAG GCTGTGGACC  
2151 CCCTGCGAAG GACAGTGGAG AAGTACAGCA TGGAGCCGGA CCCAGCAGGA  
2201 CGGGATATCT CCATCCGCCC TCTCCTGGAG CACTGTGAGA ACACCCATAT  
2251 GACCATCTGG CTTGGCATCG TCTATGCCTA CAAGGGACTT CTCATGTTGT  
2301 TCGGTTGTTT CTTAGCTTGG GAGACCCGCA ACGTCAGCAT CCCCGCACTC  
2351 AACGACAGCA AGTACATCGG GATGAGTGTG TACAACGTGG GGATCATGTG  
2401 CATCATCGGG GCCGCTGTCT CTTCTGTGAC CCGGGACCAG CCAATGTGC  
2451 AGTTCTGCAT CGTGGCTCTG CTCATCATCT TCTGCAGCAC CATCACCTC  
2501 TGCCTGGTAT TCGTGCCGAA GCTCATCACC CTGAGAACAA ACCCAGATGC  
2551 AGCAACGCAG AACAGGCGAT TCCAGTTCAC TCAGAATCAG AAGAAAGAAG  
2601 ATTCTAAAAC GTCCACCTCG GTCACCAAGT TGAACCAAGC CAGCACATCC  
2651 CGCCTGGAGG GCCTACAGTC AGAAAACCAT CGCCTGCGAA TGAAGATCAC  
2701 AGAGCTGGAT AAAGACTTGG AAGAGGTCAC CATGCAGCTG CAGGACACAC

2751 CAGAAAAGAC CACCTACATT AAACAGAACC ACTACCAAGA GCTCAATGAC  
2801 ATCCTCAACC TGGGAACTT CACTGAGAGC ACAGATGGAG GAAAGGCCAT  
2851 TTTAAAAAAT CACCTCGATC AAAATCCCCA GCTACAGTGG AACACAACAG  
2901 AGCCCTCTCG AACATGCAAA GATCCTATAG AAGATATAAA CTCTCCAGAA  
2951 CACATCCAGC GTCGGCTGTC CCTCCAGCTC CCCATCCTCC ACCACGCCTA  
3001 CCTCCCATCC ATCGGAGGCG TGGACGCCAG CTGTGTCAGC CCCTGCGTCA  
3051 GCCCCACCGC CAGCCCCCGC CACAGACATG TGCCACCCTC CTTCCGAGTC  
3101 ATGGTCTCGG GCCTGTAAGG GTGGGGGGCC TGGGCCCGGG GCCTCCCCCG  
3151 TGACAGAACC AACTGGGCA GAGGGGTCTG CTGCAGAAAC ACTGTGCGGT  
3201 CTGGCTGCGG AGAAGCTGGG CACCATGGCT GGCCTCTCAG GACCACTCGG  
3251 ATGGCACTCA GGTGGACAGG ACGGGGCAGG GGGAGACTTG GCACCTGACC  
3301 TCGAGCCTTA TTTGTGAAGT CCTTATTCT TCACAAAGAA GAGGAACGGA  
3351 AATGGGACGT CTTCTTAAC ATCTGCAAAC AAGGAGGCGC TGGGATATCR  
3401 AATTCCACCA CACTGGCGGC CCGCGTTGS TCSTAATCAT GGTCACTAAT  
3451 GTTTCCTGTG TTGAAATTGT TATCCGCTCC

## FIGURE 2

1 MASPRSSGQP GPPPPPPPPP ARLLLLLLLP LLLPLAPGAW GWARGAPRPP  
51 PSSPPLSIMG LMPLTKEVAK GSIGRGVLP VELAIEQIRN ESLLRPYFLD  
101 LRLYDTECDN AKGLKAFYDA IKYGNHLMV FGGVCPSVTS IAESLQGW N  
151 LVQLSFAATT PVLADKKKYP YFFRTVPSDN AVNPAILKLL KHYQWKRVT  
201 LTQDVQRFSE VRNDLTGVLY GEDIEISDTE SFSNDPCTSV KKLKGNDVRI  
251 ILGQFDQNMA AKVFCCAYEE NMYGSKYQWI IPGWYEPSWW EQVHTEANSS  
301 RCLRKNLLAA MEGYIGVDFE PLSSKQIKTI SGKTPQQYER EYNNKRSGVG  
351 PSKFHGYAYD GIWVIAKTLQ RAMETLHASS RHQRIQDFNY TDHTLGRILL  
401 NAMNETNFFG VTGQVVFRNG ERMGTIKFTQ FQDSREVKVG EYNAVADTLE  
451 IINDTIRFQG SEPPKDKTII LEQLRKISLP LYSILSALTI LGMIMASAFI  
501 FFNKNRNRNQK LIKMSSPYMN NLILGGMLS YASIFLFGLD GSFVSEKTFE  
551 TLCTVRTWIL TVGYTTAFGA MFAKTWRVHA IFKNVKMKKK IKDQKLLVI  
601 VGGMLLIDLC ILICWQAVDP LRRTVEKYSM EPDPAGRDIS IRPLLEHCEN  
651 THMTIWLIV YAYKGLMLF GCFLAWETR N VSIPALNDSK YIGMSVYNVG  
701 IMCIIGAAVS FLTRDQPNVQ FCIVALVIF CSTITLCLVF VPKLITLRN  
751 PDAATQNR RFQFTQNQKKED SKTSTSVTSV NQASTSRLEG LQSENHRLRM  
801 KITELDKDLE EVTMQLQDTP EKTITYIKQNH YQELNDILNL GNFTSTG  
851 KAILKNHLDQ NPQLQWNTTE PSRTCKDPIE DINSPEHIQR RLSLQLPILH  
901 HAYLPSIGGV DASCVSPCVS PTASPRHRHV PPSFRVMVSG L

Sequence: LPLLLPLAPGAWG-WARGAPRPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAIEQIRNE  
1 (signal) 1 (mature peptide)  
29 42

Other entries above 3.50

Score 11.1 at residue 39

Sequence: LLLLPLLLPLAPG-AWGWARGAPRPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAIEQI  
1 (signal) 1 (mature peptide)  
26 39

Score 8.6 at residue 38

Sequence: LLLLPLLLPLAP-GAWGWARGAPRPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAIEQ  
1 (signal) 1 (mature peptide)  
25 38

Score 8.1 at residue 35

Sequence: RLILLLLPLLLP-LAPGAWGWARGAPRPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELA  
1 (signal) 1 (mature peptide)  
22 35

Score 7.9 at residue 36

Sequence: LILLLLPLLLPL-APGAWGWARGAPRPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAI  
1 (signal) 1 (mature peptide)  
23 36

Score 6.2 at residue 9

Sequence: -QGRPPPPPPPARLILLLLPLLLPLAPGAWGWARGAPRPPSSPPLSI  
1 (signal) 1 (mature peptide)  
4 9

Score 5.7 at residue 46

Sequence: LPLAPGAWGWARG-APRPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAIEQIRNESLLR  
1 (signal) 1 (mature peptide)  
33 46

Score 5.6 at residue 747

Sequence: ITLCLVFVPKLIT-LRTNPDAATQNRRFQFTQNQKKEDSKTSTSVTSVNQASTSRLEGLQSENH  
1 (signal) 1 (mature peptide)  
734 747

Score 5.0 at residue 44

Sequence: LLLPLAPGAWGWA-RGAPRPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAIEQIRNESL  
1 (signal) 1 (mature peptide)  
31 44

Score 4.9 at residue 497

Sequence: ILSALTILGMIMA-SAFFNFKNRNQKLKMSPPYMNLIILGGMLSYSIFLFGLDGSFVSE  
1 (signal) 1 (mature peptide)  
484 497

Score 4.5 at residue 141

Sequence: LMFVGGVCPSVTS-IIAESLQGWNLVQLSFAATTPVLADKKKYPYFFRTVPSDNAVNPAILKLL<sup>3</sup>  
1 (signal) 1 (mature peptide)  
128 141

Score 4.4 at residue 734

FIGURE 3B

Sequence: FCIVALVIIFCST-ITLCLVFVPKLITLRTNPDAATQNRRFQFTQNQKKEDSKTSTSVTSVNQA  
| (signal) | (mature peptide)

721 734

Score 4.1 at residue 165

Sequence: VQLSFAATTPVLA-DKKKYPYFFRTVPSDNAVNPAILKLLKHYQWKRVGTLTQDVQRFSEVRND  
| (signal) | (mature peptide)

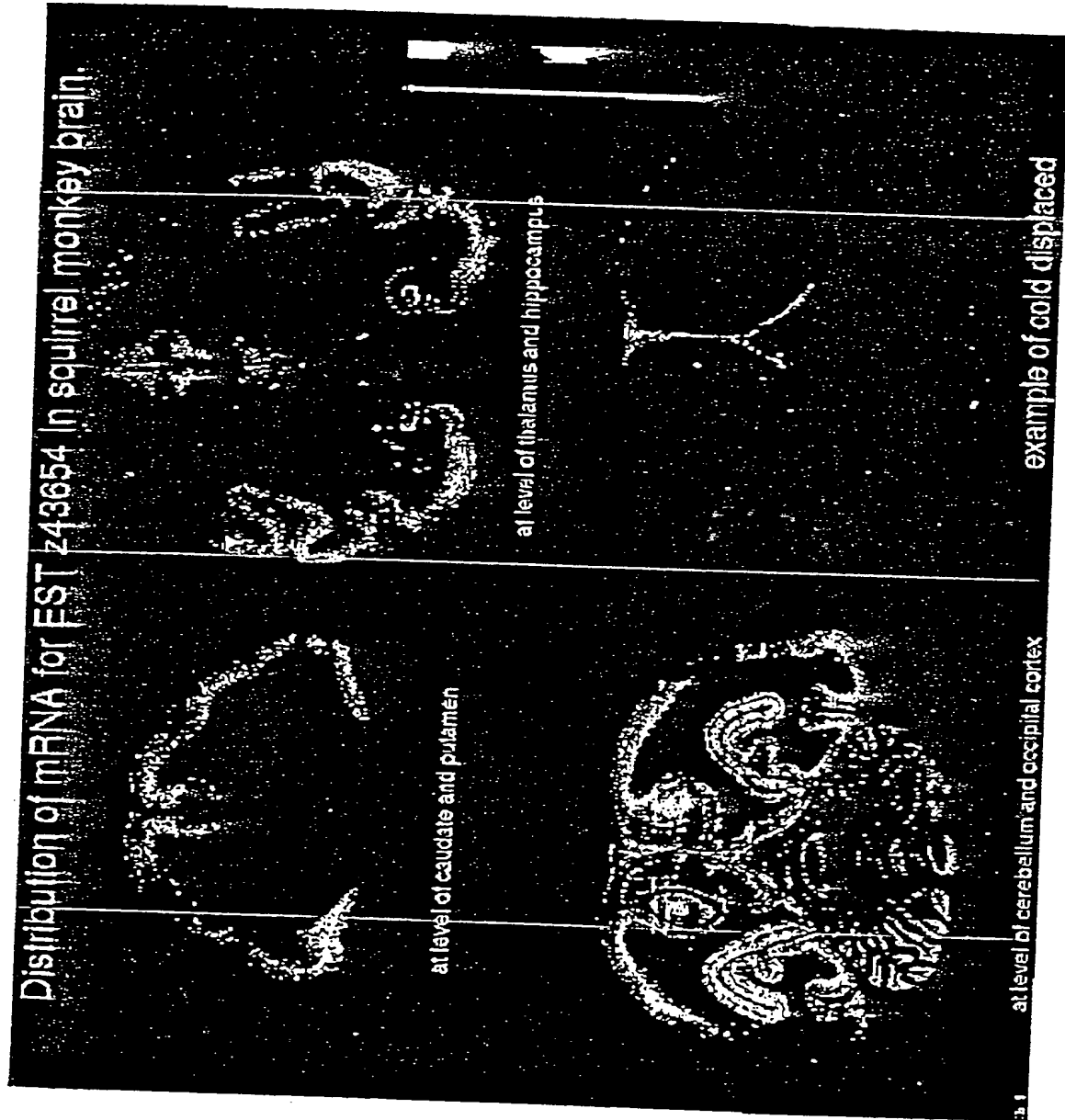
152 165

Score 3.6 at residue 158

Sequence: SLQGWNLVQLSFA-ATTPVLADKKKYPYFFRTVPSDNAVNPAILKLLKHYQWKRVGTLTQDVQR  
| (signal) | (mature peptide)

145 158

FIGURE 4

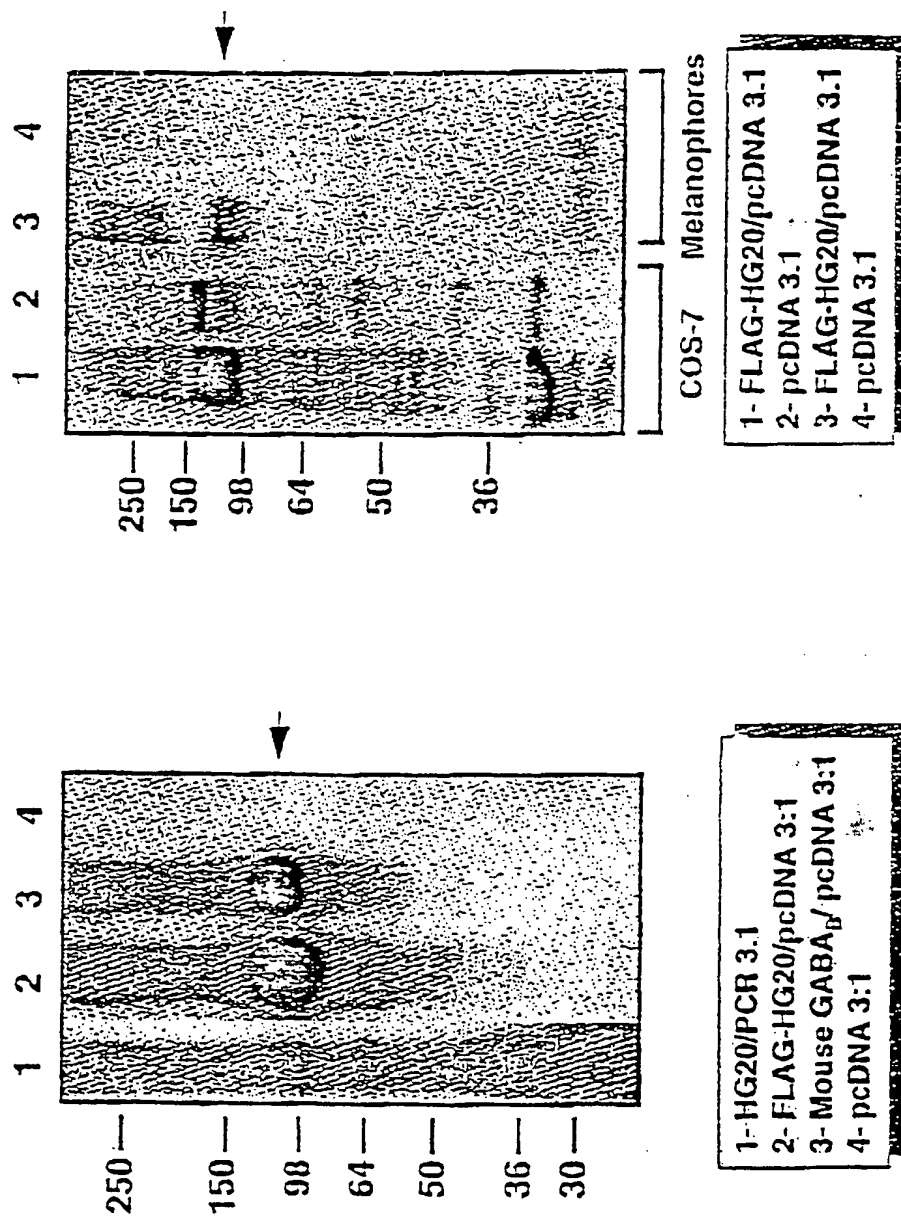




# FIGURE 5

**A** In vitro transcription/translation of metabotropic GABA<sub>B</sub>-like Receptors

**B.** Whole cell expression of GABA<sub>B</sub>-like FLAG-HG20





## FIGURE 7

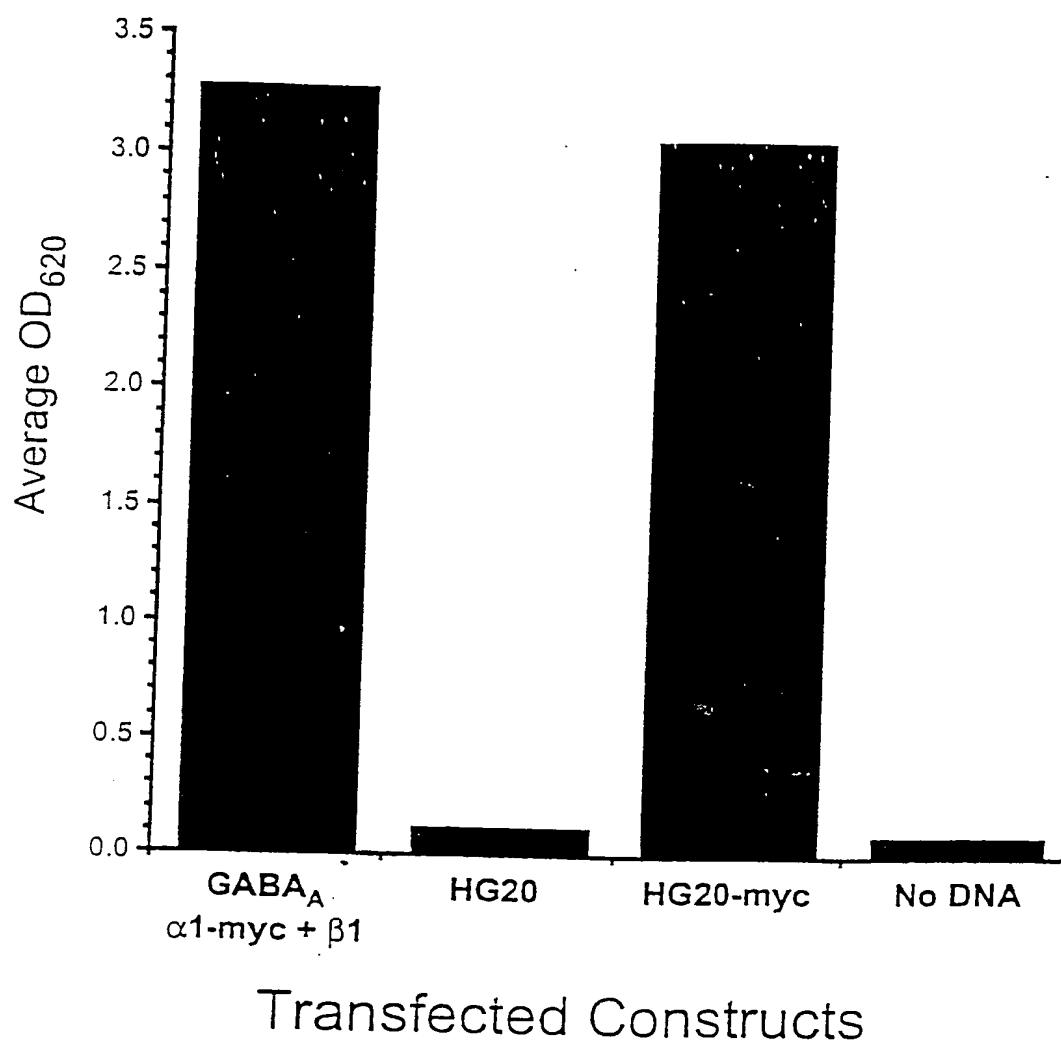
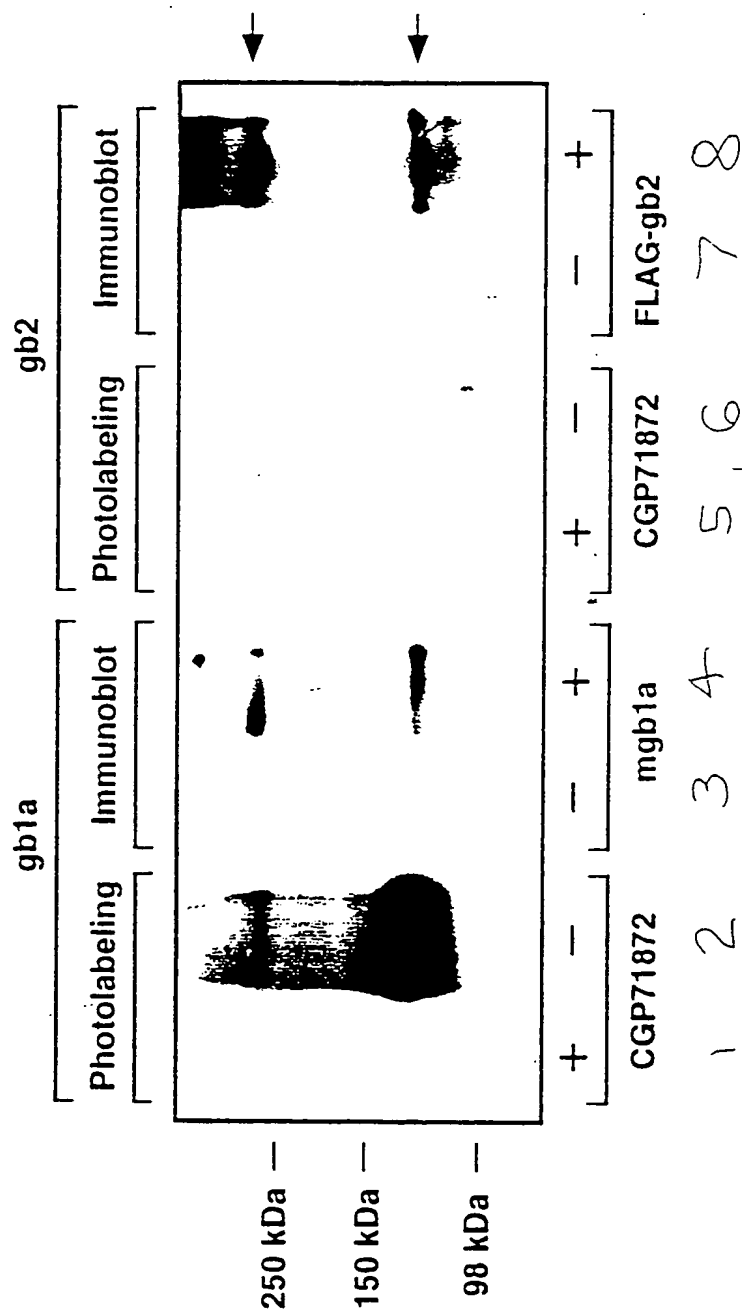


FIGURE 8

GABA-BR1b HG20 Consensus	MGPGGPCTPVGWPLPLLLVMAAGVAPVWASHSPHL---PRPHPRVPPHPSSERRAVYIGALFPMGGWP-G--GQ <sup>2</sup> MASPRSSGQGRPPPPPPPARLILLLLPLLLPLAPGAWGAPRPPSSPPSLIMGLMLPTKEVAKGSIGR M.....G.P.P.....A.....L.....R..P.P.....I..L.P.....G..G.	69 75 75
GABA-BR1b HG20 Consensus	ACQPAVEMALEDVNSRRDILPDYELKLIHDSKCPGQATKYLYELLYNDPIKIILMPG-CSSVSTLVAEARMW GVLPAVELAIEQIRN-ESLLRPYFLDLRLYDTECDNAKGLKAFYDAIKYGNHLMVFGGVCPSVTSIIAESLQGW ...PAVE.A.E.....L..Y..L.L...D..CD....K..Y.....P.....G.C.SV....AE....W	143 149 150
GABA-BR1b HG20 Consensus	NLIVLSYGSSSPALSNRQRFPTFFRTHPSATLHNPTRVKLFKKGWKKIATIQQTTEVFTSTLDDLEERVKEAGI NLVQLSFAATTPLVADKKKYPYFFRTVPSDNVNPAILKLLKHYQWKRVTLTQDVQRFEVRNDLTGVLGYGEDI NL..LS.....P..L.....P.FFRT.PS....NP...XL.....WK...T..Q....F.....DL.....I	218 224 225
GABA-BR1b HG20 Consensus	EITFRQSFSDPAVPVKNLKRQDARIIVGLFYETEARKVFCVYKERLFGKKYVWFLIGWYADNWF---KTYDPS EISDTEFSNDPCTSVKLLKGNVDRIILGQFDQNMMAKVFCCAYEENMYGSKYQWIIIPGWYEPSWVEQVHTANS EI....SF...DP...VK.LK..D.RII.G.F....A.KVFC..Y.E...G.KY.W...GWY...W....T...S	290 299 300
GABA-BR1b HG20 Consensus	INCTVEEMTEAVECHICTEIVMLNPANTRISINMSTQEFVEKLTKRLKRPETGGFQEAFLAYDAIWALALALN SRCLRNLLAAMEGYIGVDFEPLSSKQIKTISGKTPQOY-EREYNN-KRSGVGPSPKHGYAYDGIWVIATLQRA ..C.....A.EG.I.....L.....IS..T.Q....E.....KR.....F.....A..L...	365 372 375
GABA-BR1b HG20 Consensus	KTSGGGRSGVRLDFNYNNQITDQIYRAMNSSSEFVSGHVVDASGSRMAWTLIEQLQGGSYKKICYYDSTK METLHASSRHQRIQDFNYTDHTLGRILINAMNETNFFGVTTQGVVFR-NGERMGTIKFTQFQDSREVKVGEYNAVA .....R..DFNY...T....I..AMN...F.GV.G.VVF...G.RM.....Q..Q....K.G.Y....	440 446 450
GABA-BR1b HG20 Consensus	DDLWSKTDKWIGGSPADQTLVIKTRFLBQKLFISVSVLSSLGIVLAVVC---LSFNINSHVRYIQNSQPNL DTLEIINDTIRFOGSEPPKDKTII--LEQLRKISLPLYSILSALTILGMIMASAFLEFNKRNQKLIKMSPPYM D..L.....GS..P.....I.....L.....S.LS.L.I.....L.FNI.N....I..S.P..	512 519 525
GABA-BR1b HG20 Consensus	NNLTAVGCSLALAAVFPLGLDGYHIGRSQFPFVCQARLWLLGLGFLGYGSMFTKIWWVHTVFTKKEEKWRKT NNLIILGMLSYASIFLGLDGSFVSEKTFETLCTVITWILTGVYTTAFGAMFAKTWRVHA <sup>2</sup> --KQVKMK-KKI NNL...G..L..A..F..GLDG.....F...C..R.W.L..G....G.MF.K.W.VH..F..K..K....K.	587 591 500
GABA-BR1b HG20 Consensus	LEPWKLYATVGLLVGMDVLTALWQIVDPLHRTIETFAKEEPKEDIDVSLPQLEHCSSKKMNTWLGIFYGYKGL IKDQKLLVIVGMLLIDLCLICQWQAVDPLRRTVEKYSMEPDPAQRDISIRPLEHCENTHMTIWLGIYVAYKGL ...KL...VG....D...L..WQ.VDPL.RT.E....E.....D.SI.P.LEHC....M..WLG.I.Y.YRGL	662 666 675
GABA-BR1b HG20 Consensus	LLLLGIFLAYETKSVSTERKINDHRAVGMAIYNVAVLCLITAPVTMILSSQDDAFAFASLAIVFSSYITLVVLFV LMLFGCFLAWETRNVISIPALNDSKYIGMSVYVNGIMCIIGAASFLTRDQPNVQPCIVALVIFCSTITLCLVLFV L..L.G.FLA.ET..VS....ND...GM..YNV...C.I.A.V.....Q....F....L.I.F.S.ITL...FV	737 741 750
GABA-BR1b HG20 Consensus	PKMRRLITRGE-----W-----QSETQDTMKTGSS-TNNNEEEKSRL--LEKE-----NRELEKI---- PKLITLRTNPDAATQNRFPQFTQNKKEDESKTSTSVTSVNOASTSRLEGLOSSENHRLRMKITELDKDKLEEVTMQL PK...L.T.....Q.....KT..S.T..N....SRL...L..E.....LE.....	784 816 825
GABA-BR1b HG20 Consensus	--IAEKE-----ERVSE-----LRHQLQSRQQLRSRRHPPTPP--DPSGG----- QDTPEKTTYIKQNHQELNDILNLGNFTSTGGKAILKNHLDQNPQLQWNTTEPSRTCKDPFIEDINSPEHIQRR ....EK.....L...L...QL.....P....DP.....	820 891 900
GABA-BR1b HG20 Consensus	-----LPR-GPSEPPDRLSC-----DGSRVHLL---YK----- LSLQLPILHHAYLPSIGGVDASCVSPCVSPTASPRHRVPPSPFRVMVSGL .....LP..G.....C.....R.....	844 941 950

FIGURE 9



## FIGURE 10

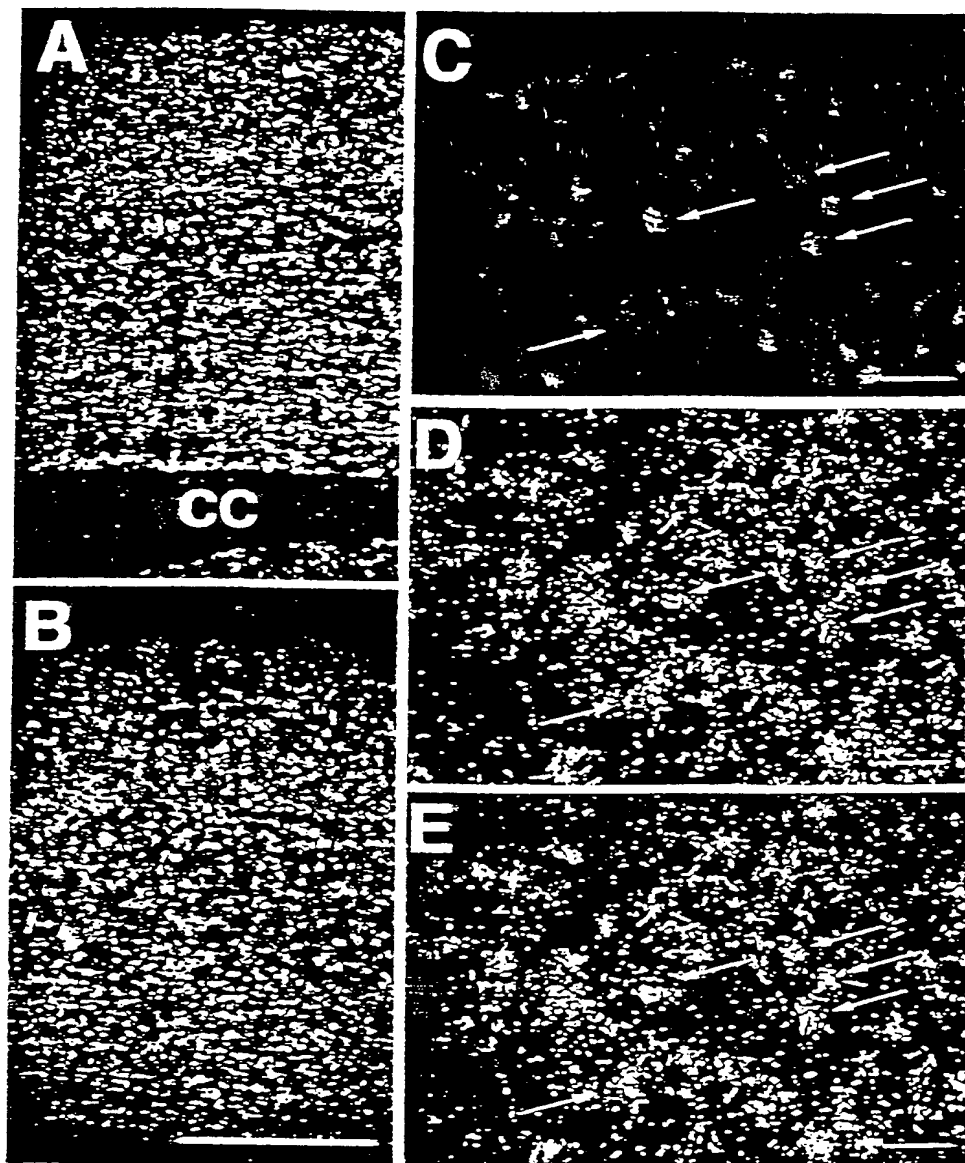


FIGURE 11

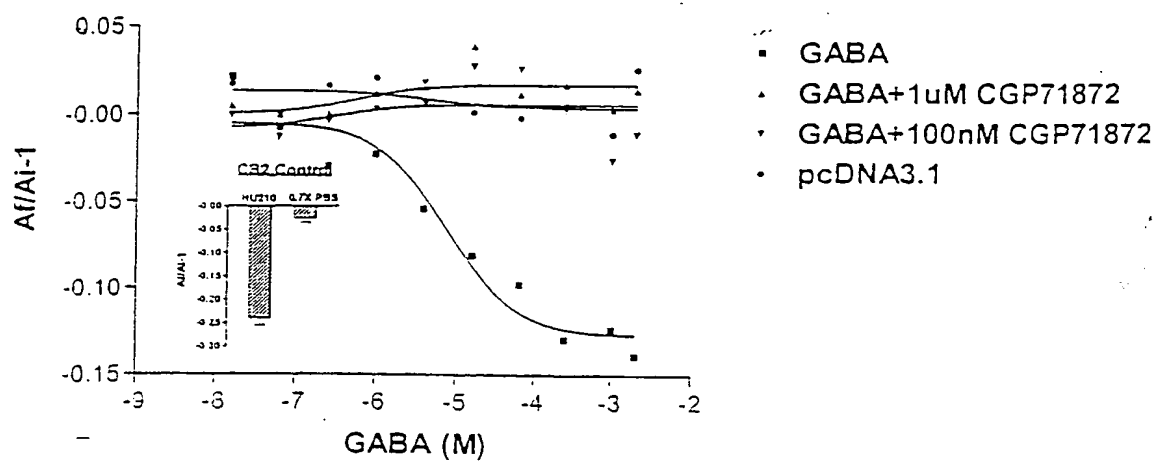
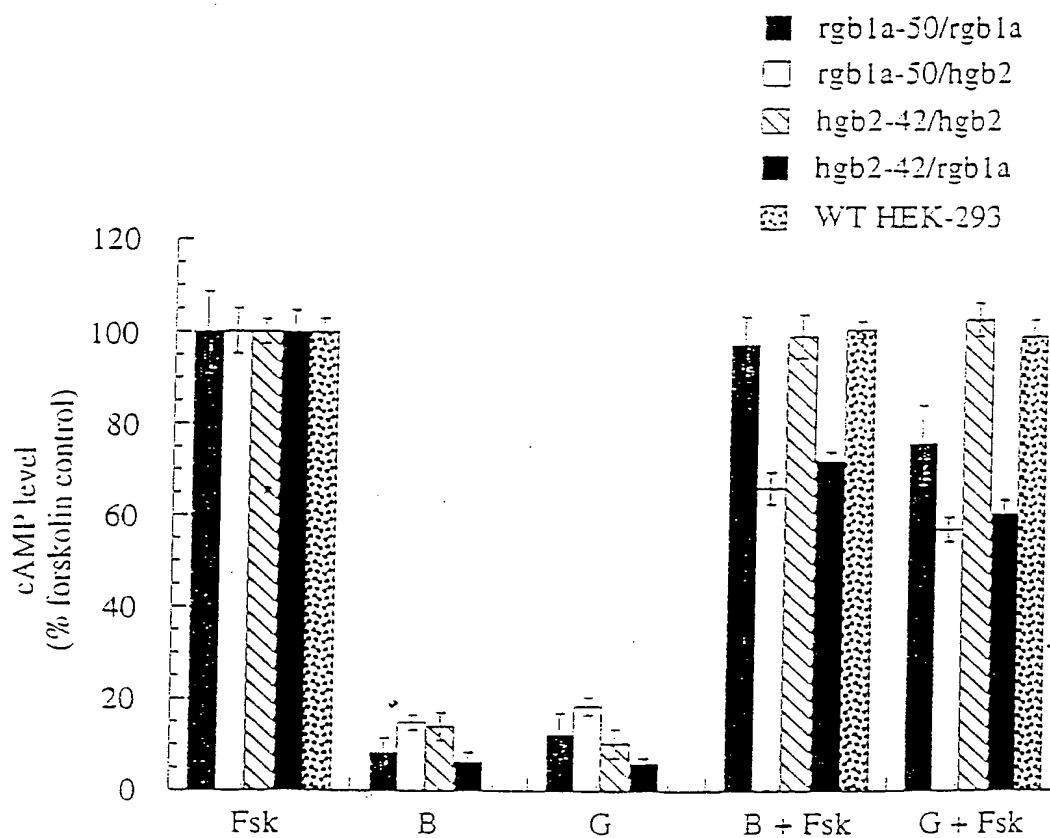


FIGURE 12





## FIGURE 13

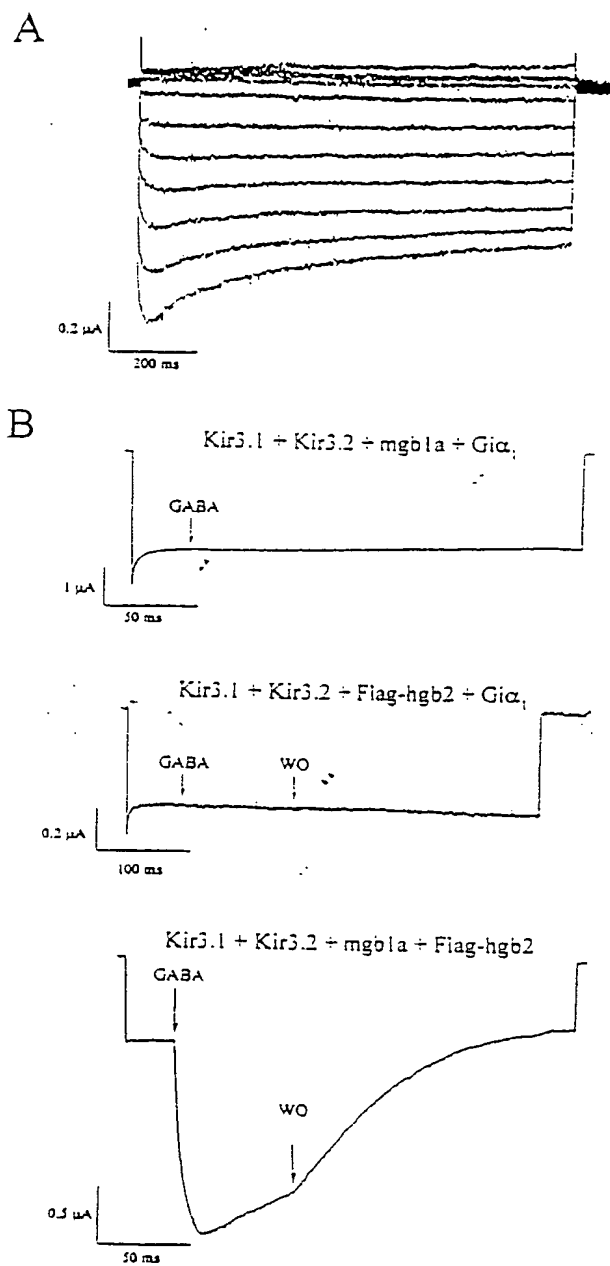


FIGURE 14

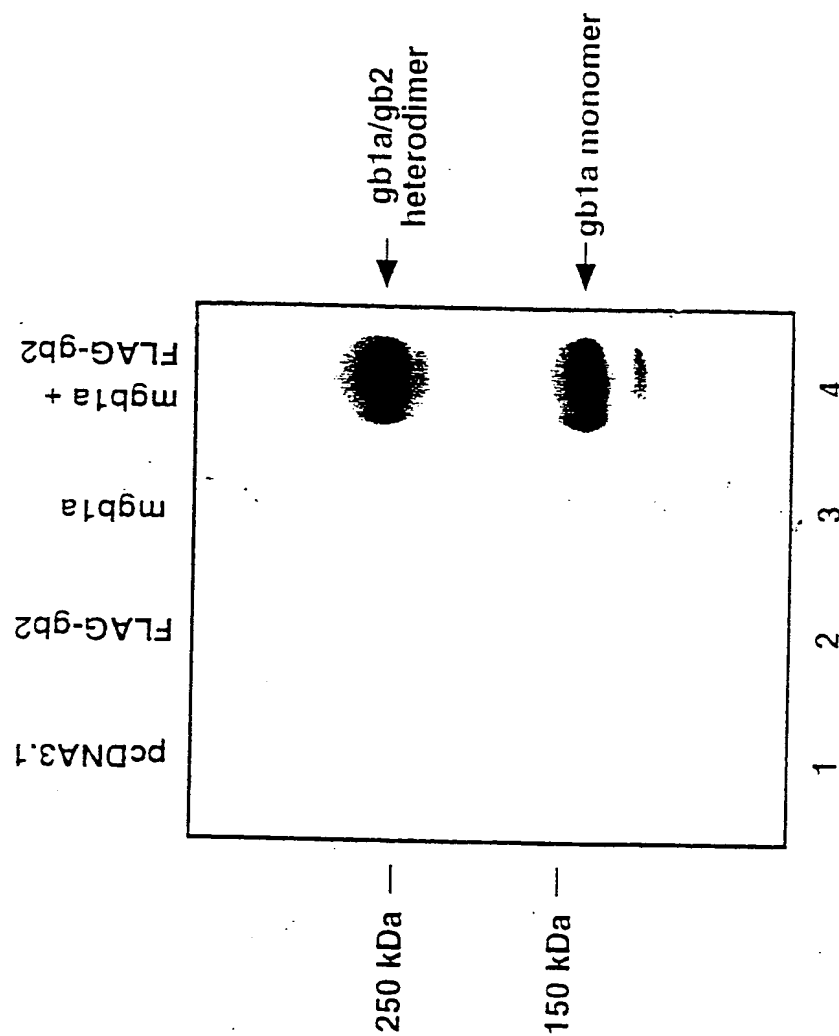


FIGURE 15

1 atgtctgtgc tgtcttctt cttctcttc ctcgcccc tgggcgtgg cggggctcag  
61 acccccaacg tcacctcgga aggttgccag attatacatc cgccctggga aggtggcatc  
121 aggtaccgtg gcttgattcg cgaccagggtg aaggccatca atttctgccc tgtggactat  
181 gagatigaat atgtgtgccg gggcgaaacg gaggtgggtgg ggcccaaggt gcgcaagtgc  
241 ctggccaacg gctcctggac ggatatggac acaccagtc gctgtgtccg aatctgctcc  
301 aagtcttatt tgacctgga aaatgggaag gtttctctga cgggtgggga cctccagct  
361 ctggatggag cccgggtgga ttccgatgt gacctgact tccatctggt gggcagctcc  
421 cggagcatct gtagtcaggg ccagtggagc accccaaagc cccactgcca ggtgaatcga  
481 acgccacact cagaacggcg tgcagtatac atcggggcgc tgttcccat gagcgggggc  
541 tggccggggg gccaggcctg ccagcctgcg gtggagatgg cgctggagga cgttaacagc  
601 cgcagagaca tctgcccga ctacgagtc aagcttatcc accacgacag caagtgcgac  
661 ccagggaag ccaccaagta cttgtatgaa ctactctaca acgacccat caagatcatc  
721 ctcatgcccg gctgcagtc tgtgtccaca ctggtagccg aggtgtcccg gatgtggaac  
781 cttattgtgc tctcatatgg ctcagctca ccagcttctg caaacgaca gcggtttcca  
841 acgttcttgc ggacacatcc atccgccaca ctcacaatc ccaccgggt gaaactcttc  
901 gaaaagtggg gctggaagaa gattgccacc atccagcaga ctaccgaggt cttcacctca  
961 aactggatg acctggagga gcgagtgaag gaggtggga ttgagatcac ttttcgacag  
1021 agtttcttct cagatccagc tgtgcctgtt aaaaacctga agcgtcaaga tgcctgaatc  
1081 atctgggac tttctatga gaccgaagcc cggaaagttt tttgtgaggt ctataaggaa  
1141 cggctcttgc ggaagaagta tctctggtt ctcacgggt ggtatgtga caactggtc  
1201 aaaacctatg accggtcaat caattgtaca gtagaagaga tgactgaggg ggtggagggc  
1261 catatcacca cggagattgt catgtgaac cctgccaaaca cccgaagcat ttccaacatg  
1321 acatcacagg aatttgtgga gaaactaacc aagcggctga aaagacacc tgaggagact  
1381 ggaggcttcc aggaggcacc actggcctat gatgtattt gggccttggc ttggccttg  
1441 aacaagacct ctggaggagg tggccgttca ggagtgcgc tggaggactt taactacaac  
1501 aaccagacca ttacagacca aatctaccgg gccatgaact cctctcctt tgagggtgtt  
1561 tctggccacg tggctttga tggcagcggc tcccgatgg catggacgt tatcgagcag  
1621 ctacagggcg gcagctacaa gaagatcggc tactacgaca gcaccaagga tgatcttcc  
1681 tggccaacaa cagacaagtg gatcgagggt tctccccag ccgaccagac cttggtcatc  
1741 aagacattcc gtttctgtc acagaaacte tttatctcc tctcagttct ctcagcctg  
1801 ggcatgttc tgtctgtgt ctgtctgtcc ttaacatct acaactccca cgtcgttat  
1861 atccagaatt cccagccaa cctgaacaat ctgactgtg tgggtgtgc actggcactg  
1921 gctgtgtct tccctctcg gctggatggt taccacatag ggagaagcca gttccgtt  
1981 gtctgccagg ccgcttctg gctcttgggc tgggcttta gctgggcta tggctctatg  
2041 ttcaccaaga tctggtgggt ccacacagtc ttcacgaaga aggaggagaa gaaggagtgg  
2101 aggaagaccc tagagccctg gaaactctat gccactgtgg gcctgtggt gggtatggat  
2161 gtctgactc ttgcatctg gcagattgtg gacctgtgc accgaacct tgagactttt  
2221 gccaggagg aaccaaagga agacatgat gtctccatc tgcctcagtt ggagcactgc  
2281 agtccaaga agatgaatac gtggcttggc atttctatg gttacaagg gctgtgtgctg  
2341 ctgtgggaa tcttcttgc ttacgaaacc aagagctgt cactgaaaa gatcaatgac  
2401 cacagggcgg tggcatggc tatctacaat gtcgcggtec tgtgtctcat cactgtctc  
2461 gtgacctga tctttccag tcagcaggac gcagccttg ctttgcctc tctggccatc  
2521 gtgttctct cctacatcac tctggtgtg ctcttctgc ccaagatgcg caggctgac  
2581 acccgagggg aatggcagtc tgaacgcag gacacatga aaacaggatc atccaccaac  
2641 aacaacgagg aagagaagtc ccgactgtt gagaaaggaaa accgagaact ggaaaagatc  
2701 atcgttgaga aagaggagcg cgtctctgaa ctgcgccatc agctccagtc tcggcagcaa  
2761 ctcgctcac ggcgccccc ccaacaccc ccagatccct ctgggggcct tccaggggga  
2821 cctctgagc ccctgaccg gcttagctgt gatggagtc gactacattt gctttacaag  
2881 tga

FIGURE 16

MLLLLLLLFLRPLGAGGAQTPNVTSEGCQIIHPPWEGGIRYRGLJRDQVKAINFLPVDY  
EIEYVCRGEREVGPKVRKCLANGSWTDMTPSRCVRIKSKSYLTLENGKVFLTGGDLPA  
LDGARVDFRCDPDFHLVGSSRSICSQGQWSTPKPHCQVNRTPHSERRAVYIGALFPMSGG  
WPGGQACQPAVEMALEDVNSRRDILPDYELKLIHHD SKCDPGQATKYLYELLYNDPIKII  
LMPGCSSVSTLVAEAARMWNLI VLSYGSSSPALSNRQRFPTFFRTHPSATLHNPTRVKLF  
EKWGWKKIATIQQTTEVFTSTLDDLEERVKEAGIEITFRQSFFSDPAVPVKNLKRQDARI  
IVGLFYETEARKVFCEVYKERLFGKKYVWFLIGWYADNWFKTYDPSINCTVEEMTEAVEG  
HITTEIVMLNPANTRISISNMTSQEFVEKLT KRLKRHP EETGGFQEAPLAYDAIWALALAL  
NKTSGGGGRSGVRLEDFNYNNQTTTDDQIYRAMNSSSFEGVSGHVFDASGSRMAWTJIEQ  
LQGGSYKKIGYYDSTKDDLSWSKTDKWIGGSPPADQTLVIKTRFLSQKLFISVSVLSSL  
GIVLAVVCLSFNIYN SHARYIQNSQPNLNNLTAVGCSLALAVFPLGLDGYHIGRSQFPF  
VCQARLWLLGLGFSLGYGSMFTKIWWVHTVFTKKEEKKEWRKTLPEWKLYATVGLLVGMD  
VLT LAIWQIVDPLHRTIETFAKEE PKEDIDVSILPQLEHCSSKKMNTWLGIFYGYKGLLL  
LLGIFLAYETKSVSTEKINDHRAVGMAIYNVAVLCLITAPVTMILSSQQDAAFASLAI  
VFSSYITLVVLFVPKMRRLLITRGEWQSETQDTMKTGSSTNNNEEEKSRLLKENRELEKI  
IAEKEERVSEL RHQLQSRQQLRSRRHPPTPPDPSGGLPRGPSEPPDRLS CDGSRVHLLYK

# FIGURE 17

## [<sup>125</sup>I]CGP71872 labeling of the GABA<sub>B</sub> receptor N-terminal domain

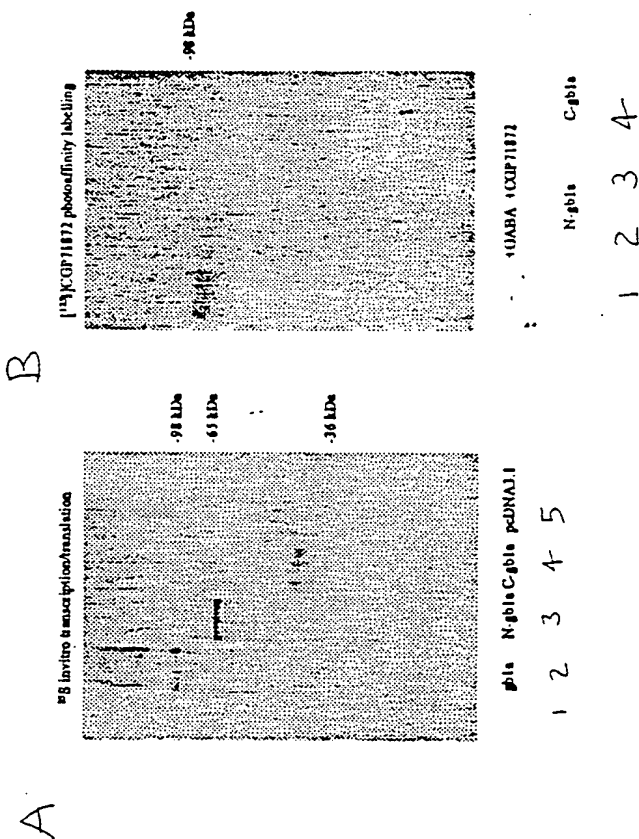


FIGURE 18A

MLLLLLLAPLFLRPPGAGGAHTPNATSEGCQIIHPPWEGGIRYRGLTRDQV  
KAINFLPVDYIEYVCRGEREVVGPVKRCLANGSWTDMDTPSRCVRICS  
KSYLTLENGKVFLTGGDLPALD GARADFRCDPDFHLVGSSRSICSQGGQWST  
PKPHCQVNRTPHSERRAVYIGALFPMMSGGWPGGQACQPAVEMALEDVNS  
RRDILPDYELKLIHHD SKCDPGQATKYLYELLYNDPIKIILMPGCSSVSTLV  
AEAARMWNLIVLSYGSSSPALSNRQRFPTFFRTHPSATLHNPTRVKLFEKW  
GWKKIATIQQTTEVFTSTLDDLEERVKEAGIEITFRQSFFSDPAVPVKNLKRQ  
DARIIVGLFYETEARKVFCEVYKERLFGKKYVWFLIGWYADNWFKIYDPS  
INCTVDEMTEAVEGHITTEIVMLNPANTRISISNMTSQEFVEKLT KRLKRHPE  
ETGGFQEAPLAYDAIWALALALNKTSGGGGRSGVRLED FNYNNTITDQI  
YRAMNSSFEGVSGHVVF DASGSRMAWTLIEQLQGGSYKKIGYYDSTKDD  
LSWSKTDKWIGGSPPADQTLVIKTRFLSQKLFISVSVLSSLGIVLAVVCLSF  
NIYNSHVRYIQNSQPNLNNLTAVGCSLALAAVFPLGLDG YHIGRNQFPFV  
CQARLWLLGLGFS LGYGSMTFKIWWVHTVFTKKEEKKEWRKTLEPWKLY  
ATVGLLVGMDVLT LAIWQIVDPLHRTIETFAKEEPKEDIDVSILPQLEHCSS  
RKMNTWLGIFYGYKGLLLLLLGIFLAYETKSVSTEKINDHRAVGMAIYNVA  
VLCLITAPVTMILSSQQDAAFASFASLAIVFSSYITLVVLFVPKMRR LITRGE  
WQSEAQDTMKTGSSTNNNEEEKSR LLEKENRELEKIIAEKEERVSEL RHQLQ  
SRQQLRSRRHPPTPPEPSGGLPRGPPEPPDRLSCDGSRVHLLYK

FIGURE 18B

1 atgttgctgc tgcctact ggcgccactc ttctccgcc ccccgggcgc gggcggggcg  
 61 cataccccca acgccacctc agaagggtgc cagatcatc acccgccctg ggaagggggc  
 121 atcaggtacc ggggectgac tggggaccag gtaaggcta tcaacttct gccagtggac  
 181 tatgagatg agtatgtgtg ccggggggag cgcgaggtgg tggggccca ggtccgcaag  
 241 tgcctggcca acggctctg gacagatg gacacacca gccgctgtgt ccgaatctgc  
 301 tccaagtct attgacctt ggaaaatggg aaggtttcc tgacgggtgg ggacctccca  
 361 gctctggacg gagcccgggc ggatttccgg tggaccccg acttccatc ggtggggcgc  
 421 tcccgagca tctgtatga gggccagtgg agcaccacca agccctactg ccaggtgaat  
 481 cgaacgccac actcagaacg gcgcgcagtg tacatcgggg cactgttcc catgagcggg  
 541 ggctggccag gggggccagg ctggccagccc gcggtggaga tggcgctgga ggacgtgaat  
 601 agccgcaggg acatcctgcc ggactatgag ctcaagctca tccaccaga cagcaagtgt  
 661 gatccaggcc aagccacca gtacctatg gagctgtct acaacgacct tatcaagatc  
 721 atcctatgc ctggctgcag ctctgtctcc acgctgggtg ctgaggctgc taggatgtgg  
 781 aacctcatg tgccttcta tggctccagc tcaccagccc tgcataaccg gcagcgttcc  
 841 cccactttct tccgaacgca cccatcagcc acactccaca accctaccgg cgtgaaactc  
 901 ttgaaaagt ggggctggaa gaagattgct accatccagc agaccactga ggtcttact  
 961 tgcactctgg acgacctgga ggaacgagt aaggaggctg gaattgagt tactttccgc  
 1021 cagagtttct tctcagatcc agctgtgccc gtaaaaaacc tgaagcgcca ggatcccca  
 1081 atcactgtgg gactttcta tgagactgaa gcccgaaag ttttttga ggtgtacaag  
 1141 gagcgtctct tgggaagaa gtactgtggt ttctcattg ggtggtatgc tgacaattgg  
 1201 ttcaagatct acgaccttc tatcaactgc acagtggatg agatgactga ggcggtggag  
 1261 ggccacatca caactgagat tgcctgtctg aatcctgcca ataccgcag catttccaac  
 1321 atgacatccc aggaattgt ggagaaacta accaagcgac tgaagagaca ccttgaggag  
 1381 acaggaggct tccaggaggc accgctggcc tatgatgcca tctgggctt ggcactggcc  
 1441 ctgaacaaga catctggagg aggcggcctg tctgtgtgc gccctggagga ctcaactac  
 1501 aacaaccaga ccattaccga ccaaatctac cgggcaatga actcttctc cttgagggt  
 1561 gtctctggcc atgtgtgtt tgatgccagc ggcctcggga tggcatggac gcttatcgag  
 1621 cagcttcagg gtggcagcta caagaagt ggctactarg acagcaccaa ggatgattct  
 1681 tcttggcca aacagataa atggattga gggcccccc cagctgacca gacctgtgtc  
 1741 atcaagacat tccgcttct gtcacagaaa ctcttattc cgtctcagt tcttccagc  
 1801 ctgggcattg tctagctgt tctgtgtg tctttaaca tctacaactc acatgtcgt  
 1861 tataccaga actcacagcc caacctgaac aacctgactg ctgtgggtg ctactggct  
 1921 ttgctgtg tcttccccct ggggctgat ggttaccaca ttggaggaa ccagtttct  
 1981 ttgctgtg aggcctgct ctggctcctg ggcctgggt ttagtctggg ctacggttcc  
 2041 atgttacca agattgggt ggtccacacg gtcttccaa agaaggaaga aaagaaggag  
 2101 tggaggaaaga ctctggaacc ctggaagctg tatgccacag tgggctgct ggtgggcat  
 2161 gatgtctca ctctgcca ctggcagatc gtggaccctc tgcaccggac cattgagaca  
 2221 ttgccaagg aggaacctaa ggaagatant gacgtctcta ttctcccca gctggagcat  
 2281 tgcagctcca ggaagatgaa tacatggctt ggcatttct atggttaca ggggctgctg  
 2341 ctgctgtg gaaatctct tgcattgag accaagagt tgcactga gaagatcaat  
 2401 gatcaccggg ctgtgggcat ggctatctac aatgtggcag tctgtgct catcactgt  
 2461 cctgtacca tgattctgc cagccagcag gatgcagct ttgccttgc ctcttggc  
 2521 atagtttct cctctatat cacttgtt gtgtctttg tgcccaagat gcgcaggctg  
 2581 atcaccgag gggaaatgca gtggaggcg caggacacca tgaagacagg gtcacgacc  
 2641 aacaacaacg aggaggagaa gtcccggtg ttggagaagg agaaccgtga actggaaaag  
 2701 atcattgtg agaaagagga gcgtgtctt gaactgcgc atcaactcca gtctggcag  
 2761 cagctcgt cccggcgcca cccaccgaca ccccagaac ccttggggg cctgccagg  
 2821 ggacccccct agcccccca cggcctagc tgtgatgga gtcgagtga ttgcttat  
 2881 aagtga

FIGURE 19A

1 atgtgtgtgc tgcgtgtggc gccactcttc ctccgcccc cgggctgggg cggggcgag  
61 acccccaacg ccaccicaga aggttgccag atcatacacc cgccctggga agggggcatc  
121 aggtaccggg gctgtactcg ggaccagggtg aaggctatca acttctgtcc agtggactat  
181 gagantgagt atgtgtgccg gggggagcgc gaggtgggtg ggcccaaggt ccgcaagtgc  
241 ctggccaacg gctcctggac agatatggac acaccagcc gctgtgtccg aatctgtcc  
301 aagtcttatt tgaccctgga aatgggaag gtttctga cgggtgggga cctccagct  
361 ctggacggag cccgggtgga ttccgggtgt gaccccgact tccatctggt gggcagctcc  
421 cggagcatct gtagtcaggg ccagtggagc accccaagc cccactgcca ggtgaatcga  
481 acgccacact cagaacggcg cgcagtgtac arcggggcac tgttcccat gagcgggggc  
541 tggccagggg gccaggcctg ccagcccgcg gtggagatgg cgttgggga cgtgaatagc  
601 cgcagggaca tctgtccgga ctatgagctc aagctcacc accacgacag caagtgtgat  
661 ccaggccaag ccaccaagta cctatatgag ctgtctaca acgacctat caagatcatc  
721 cttatgctg gctgcagctc tgtctccag ctgggtggctg aggtgtggaac  
781 ctcatgtgc ttctctatg ctccagctca ccagccctgt caaacggga cgtttccc  
841 actttctcc gaacgcacc atcagccaca ctccacaacc ctaccgcgt gaaactctt  
901 gaaaagtggg gctggaagaa gattgctacc atccagcaga cactgaggt ctactctg  
961 actctggacg acctgggga acgagtgaag gaggtggaa ttgagatc ttccgccag  
1021 agttctctc cagatccagc tgtccccg ctaaacctga agcggcaga tggccgaatc  
1081 atctgtggac ttctatga gactgaagcc cggaaagtt ttgtgaggt gtacaaggag  
1141 cgtctcttg ggaagaagta cgtctggtc ctatgtgtg ggtatgtga caattgttc  
1201 aagatctacg accctctat caactgcaca gtggatgaga tgactaggc ggtggagggc  
1261 cacatcaca ctgagattgt catgtgaat cctgccaata cccgcagcat ttccaatc  
1321 acatcccagg aattgtgga gaaactaacc aagcgactga aaagacacc tgaggagaca  
1381 ggaggcttcc aggaggcacc gctggcctat gatgccatc gggcctggc actggccctg  
1441 aacaagacat ctggaggagg cggccgtct ggtgtgccc tggaggactt caactacaac  
1501 aaccagacca ttaccgacca aatctaccgg gcaatgaact ctctgctt ttgggtgtc  
1561 tctggccatg tgggtttga tggcagcgc tctggatgg catggacgt tatcgagcag  
1621 cctcagggtg gcagctaca gaagattggc tactatgaca gcaccaagga tgatcttcc  
1681 tggccaaaa cagataaatg gattggaggg tccccccag ctgaccagac cctgttcatc  
1741 aagacattcc gcttctgtc acagaaactc ttatctccg tctcagttc ctccagctg  
1801 ggcartgtcc tagctgtgt ctgtctgtc ttaacatct acaactcaca tgtccgttat  
1861 atccagaact cacagcccaa cctgaacaac ctgactgtg tgggtgtc actggctta  
1921 gctgtgtct tccccctgg gctcgatgt taccacatt ggaggaaaca gtttcttc  
1981 gtctgccagg cncgctctg gctcctggc ctgggctta gcttggcta cggttccatg  
2041 ttaccaaga ttgtgtgggt ccacacggc ttacaaaga aggaagaaa gaaggagtgg  
2101 aggaagactc tggaaacctg gaagctgtat gccacagtgg gctgtgtgt gggcattgat  
2161 gtctcactc tgcctatct gcagatctg gacctctgc accggacct tgagacatt  
2221 gccaaaggag aacctaaagg agatattgac gtctctatc tggccagct ggagcattg  
2281 agctccagga agatgaatc atggcctggc atttctatg gttacaagg gctgtgtct  
2341 ctgtgggaa tcttcttgc ttatgagacc aagagtgtt ccactgaga gatcaatgat  
2401 caccgggctg tgggcatggc tatctacaat gtggcagtc tgtgtctcat cactgtctc  
2461 gtacccatga ttctgtccg ccagcaggat gcagccttg ctttgcctc tcttgccata  
2521 gtttctct cctatacac tctgtgtg ctcttctg ccaagatgc caggctgatc  
2581 acccgagggg aatggcagtc ggaggcgag gacaccatga agacagggtc atcgaccaac  
2641 aacaacgagg aggagaagtc ccgctgttg gagaaggaga accgtgaact ggaagatc  
2701 attgtgaga aagaggagcg tctctgaa ctgcgccatc aactccagtc tggcagcag  
2761 ctccgtctcc ggcgccacc accgacacc ccagaacct ctgggggct gccagggga  
2821 cccctgagc ccccgaccg gctagctgt gatgggagtc gattgcatc gcttataag  
2881 tgagggtagg gtgaggagg acaggccagt agggggagg aaaggagag ggaagggga  
2941 ggggactcag gaagcagggg gtcccatcc ccagctggga agaactgtct atcaatctc  
3001 atctctgta aatcatgtc cccctgtg ttctgggtg atttgggtc ctcatacctc  
3061 tgggaaacag acctttct ctctactgc ttcatgtaatt ttgtatcac ctctcaca



## FIGURE 19B

3121 tttagttcgt acctggcttg aagctgctca ctgctcacac gctgectect cagcagcctc  
3181 actgcatctt tctctccca tgcaacaccc tcttctagtt accacggcaa cccctgcagc  
3241 tctctgcct ttgtgtctg ttctgtcca gcaggggtct cccaacaagt gctcttcca  
3301 ccccaaaggg gcctctctt ttctccactg tcataatctc ttccatctt acctgccctt  
3361 ctatacttc tcacatgtgg ctccccctga atttgcttc cttggggagc tcaattttt  
3421 cgccaaggtt cacatgtccc ttgectctgc tctgtgcaact cagctcagc acacatgcat  
3481 cctccccctt cctgcgtgtg cccactgaac atgctcatgt gtacacacgc ttcccgta  
3541 tgcctctc atgttcagtc acatgtgtc tcgggtgccc tgcattcaca gctacgtgtg  
3601 cccctctcat ggatcatgggt ctgcccttga gcgtgttgg gtaggcagt gcaattgtc  
3661 tagcatgtg agtcatgtct ttctattg cacacgtcca tgmattcca tgtacttcc  
3721 ctgtgtacc tccatgtacc ttgtgtact tctccctta aatcatgtta ttctctgac  
3781 agagccat atgtacctacc ctgcacatg ttatgcact ttcccaatt catgtttgt  
3841 gggggccatc acacctctc ctgtcacag aatctcatt tctgtcaga ttcccccat  
3901 ctccattgca ttcatgtact acctcagtc tacactcaca atcaattct cccaagactg  
3961 ctccctttg ttgtgttt tttaggggg aattaaggaa aaataagtgg gggcaggtt  
4021 ggagagctgc ttccagtga tagttgatga gaatcctgac caaagggaagg caccctgac  
4081 tgttgggata gacagatgga cctatggggt gggaggtggt gtcccttca cactgtgtg  
4141 tctctgggg aaggatctc ccgaatcica ataaaccagt gaacagtgtg actcggaaaa  
4201 aaaaaaaaa aaaaaaaaa

# FIGURE 20

| proximal to HSN-1, FCMD, DYS loci on chromosome 9

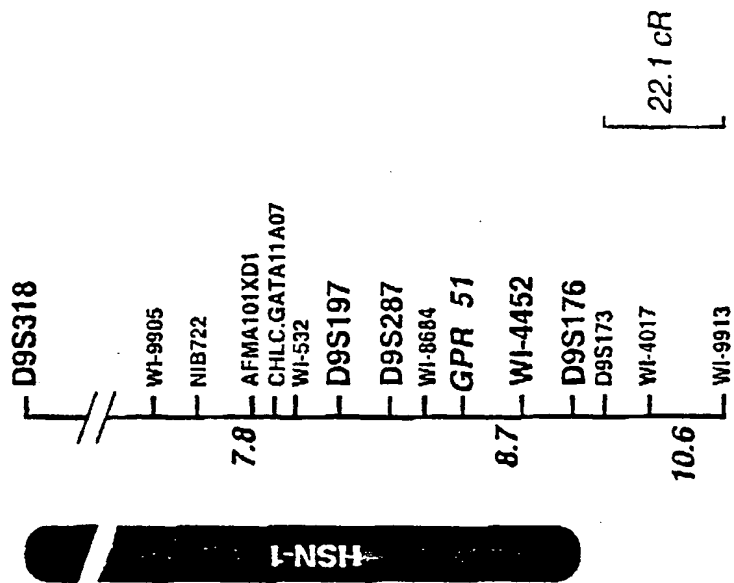


FIGURE 21

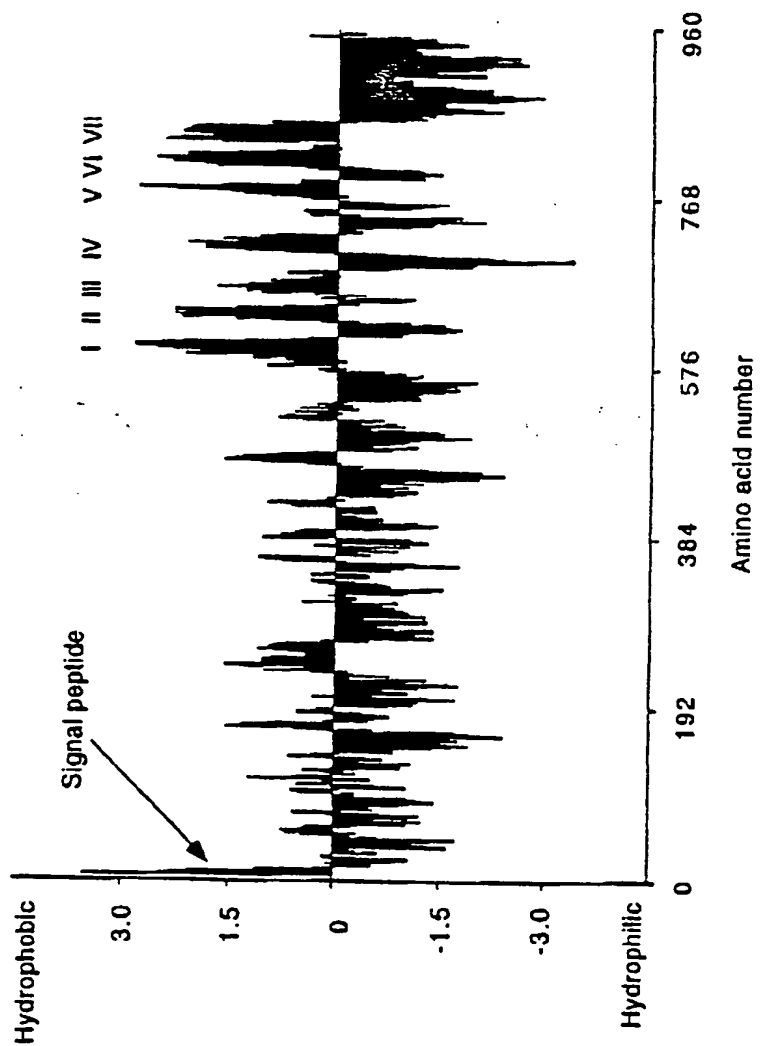
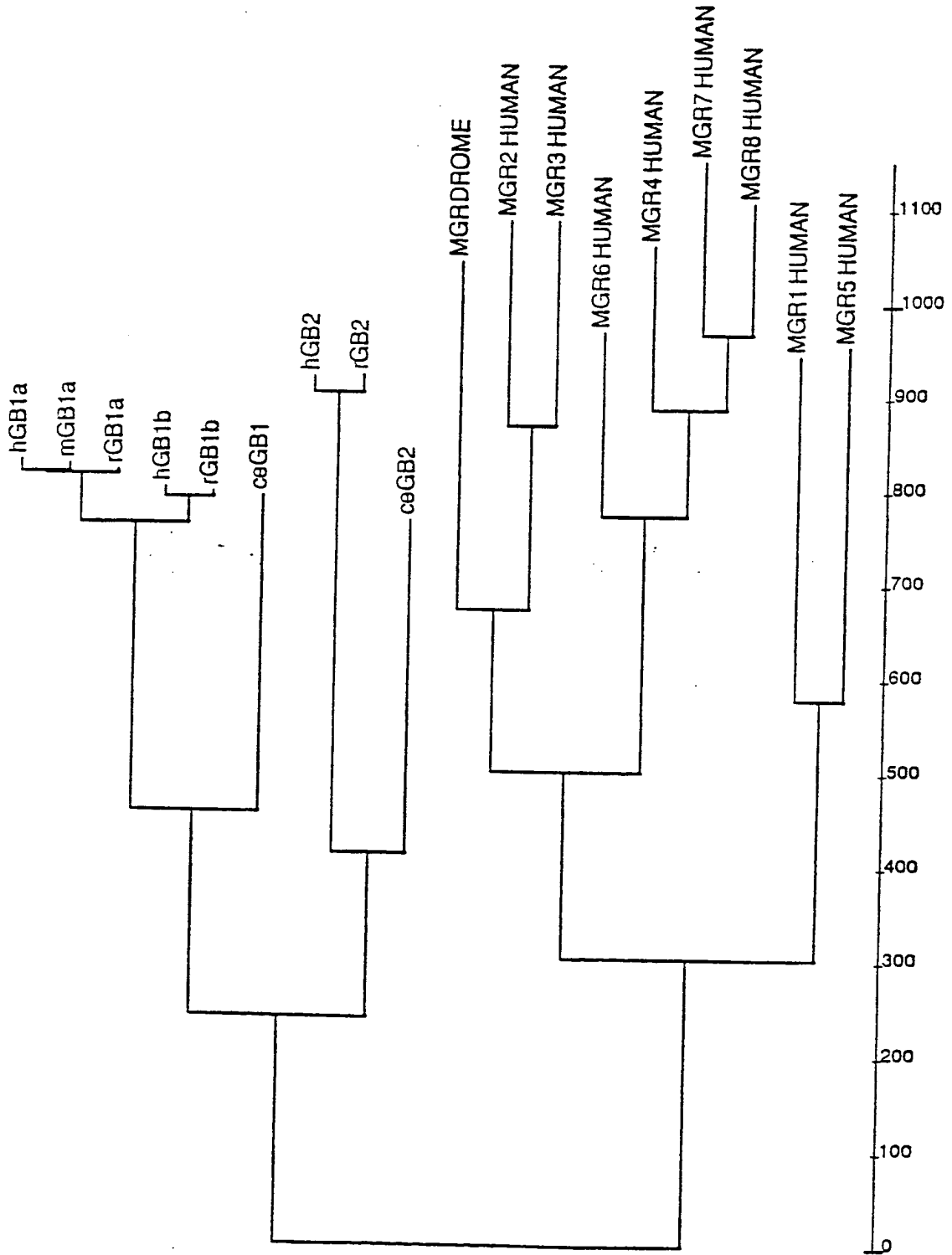


FIGURE 22



# FIGURE 23

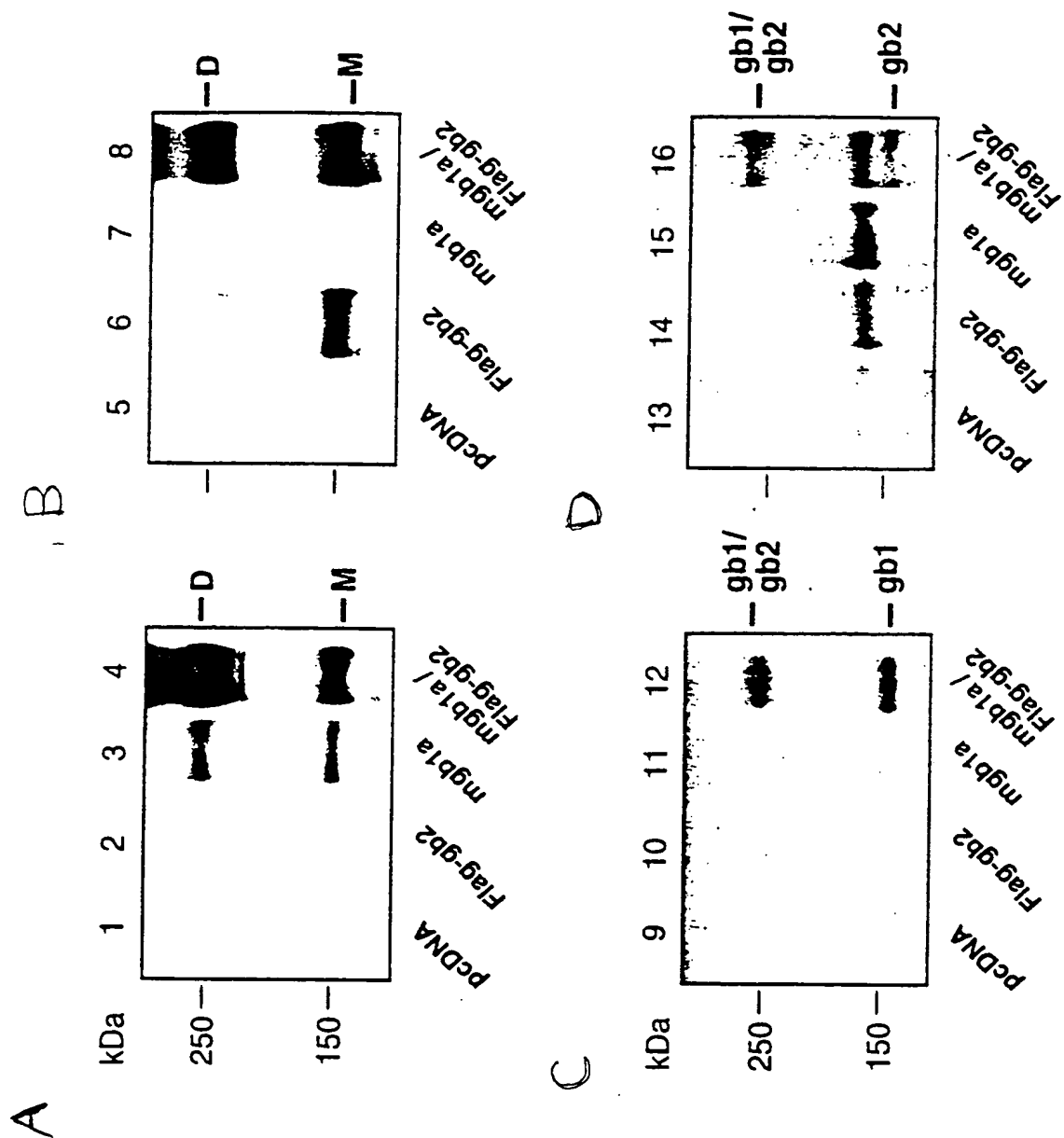
Coiled-coil domain in C-terminus of gbl a and HG20 mediating heterodimerization

gbl ...KCSFA.QDTHKTCSTNNREK...SRLEK..ENRELEKIINAEKEEVSELRHQLQSRQQLRSHRPP  
 HG20 QNRRFCTQNQKKEDSKTSTSVTGVNQASTSRLEGLOSENHLRMKLTLEOKDLEVTGQLQDTPENTYINQ

FIGURE 24

CVSPCVSPTASPRHRHVPPSF RVMV SGL

FIGURE 25



1  
 2  
 3  
 4  
 5  
 6  
 7  
 8  
 9  
 10  
 11  
 12  
 13  
 14  
 15  
 16  
 17  
 18  
 19  
 20  
 21  
 22  
 23  
 24  
 25  
 26  
 27  
 28  
 29  
 30  
 31  
 32  
 33  
 34  
 35  
 36  
 37  
 38  
 39  
 40  
 41  
 42  
 43  
 44  
 45  
 46  
 47  
 48  
 49  
 50  
 51  
 52  
 53  
 54  
 55  
 56  
 57  
 58  
 59  
 60  
 61  
 62  
 63  
 64  
 65  
 66  
 67  
 68  
 69  
 70  
 71  
 72  
 73  
 74  
 75  
 76  
 77  
 78  
 79  
 80  
 81  
 82  
 83  
 84  
 85  
 86  
 87  
 88  
 89  
 90  
 91  
 92  
 93  
 94  
 95  
 96  
 97  
 98  
 99  
 100  
 101  
 102  
 103  
 104  
 105  
 106  
 107  
 108  
 109  
 110  
 111  
 112  
 113  
 114  
 115  
 116  
 117  
 118  
 119  
 120  
 121  
 122  
 123  
 124  
 125  
 126  
 127  
 128  
 129  
 130  
 131  
 132  
 133  
 134  
 135  
 136  
 137  
 138  
 139  
 140  
 141  
 142  
 143  
 144  
 145  
 146  
 147  
 148  
 149  
 150  
 151  
 152  
 153  
 154  
 155  
 156  
 157  
 158  
 159  
 160  
 161  
 162  
 163  
 164  
 165  
 166  
 167  
 168  
 169  
 170  
 171  
 172  
 173  
 174  
 175  
 176  
 177  
 178  
 179  
 180  
 181  
 182  
 183  
 184  
 185  
 186  
 187  
 188  
 189  
 190  
 191  
 192  
 193  
 194  
 195  
 196  
 197  
 198  
 199  
 200  
 201  
 202  
 203  
 204  
 205  
 206  
 207  
 208  
 209  
 210  
 211  
 212  
 213  
 214  
 215  
 216  
 217  
 218  
 219  
 220  
 221  
 222  
 223  
 224  
 225  
 226  
 227  
 228  
 229  
 230  
 231  
 232  
 233  
 234  
 235  
 236  
 237  
 238  
 239  
 240  
 241  
 242  
 243  
 244  
 245  
 246  
 247  
 248  
 249  
 250  
 251  
 252  
 253  
 254  
 255  
 256  
 257  
 258  
 259  
 260  
 261  
 262  
 263  
 264  
 265  
 266  
 267  
 268  
 269  
 270  
 271  
 272  
 273  
 274  
 275  
 276  
 277  
 278  
 279  
 280  
 281  
 282  
 283  
 284  
 285  
 286  
 287  
 288  
 289  
 290  
 291  
 292  
 293  
 294  
 295  
 296  
 297  
 298  
 299  
 300  
 301  
 302  
 303  
 304  
 305  
 306  
 307  
 308  
 309  
 310  
 311  
 312  
 313  
 314  
 315  
 316  
 317  
 318  
 319  
 320  
 321  
 322  
 323  
 324  
 325  
 326  
 327  
 328  
 329  
 330  
 331  
 332  
 333  
 334  
 335  
 336  
 337  
 338  
 339  
 340  
 341  
 342  
 343  
 344  
 345  
 346  
 347  
 348  
 349  
 350  
 351  
 352  
 353  
 354  
 355  
 356  
 357  
 358  
 359  
 360  
 361  
 362  
 363  
 364  
 365  
 366  
 367  
 368  
 369  
 370  
 371  
 372  
 373  
 374  
 375  
 376  
 377  
 378  
 379  
 380  
 381  
 382  
 383  
 384  
 385  
 386  
 387  
 388  
 389  
 390  
 391  
 392  
 393  
 394  
 395  
 396  
 397  
 398  
 399  
 400  
 401  
 402  
 403  
 404  
 405  
 406  
 407  
 408  
 409  
 410  
 411  
 412  
 413  
 414  
 415  
 416  
 417  
 418  
 419  
 420  
 421  
 422  
 423  
 424  
 425  
 426  
 427  
 428  
 429  
 430  
 431  
 432  
 433  
 434  
 435  
 436  
 437  
 438  
 439  
 440  
 441  
 442  
 443  
 444  
 445  
 446  
 447  
 448  
 449  
 450  
 451  
 452  
 453  
 454  
 455  
 456  
 457  
 458  
 459  
 460  
 461  
 462  
 463  
 464  
 465  
 466  
 467  
 468  
 469  
 470  
 471  
 472  
 473  
 474  
 475  
 476  
 477  
 478  
 479  
 480  
 481  
 482  
 483  
 484  
 485  
 486  
 487  
 488  
 489  
 490  
 491  
 492  
 493  
 494  
 495  
 496  
 497  
 498  
 499  
 500  
 501  
 502  
 503  
 504  
 505  
 506  
 507  
 508  
 509  
 510  
 511  
 512  
 513  
 514  
 515  
 516  
 517  
 518  
 519  
 520  
 521  
 522  
 523  
 524  
 525



## FIGURE 26B

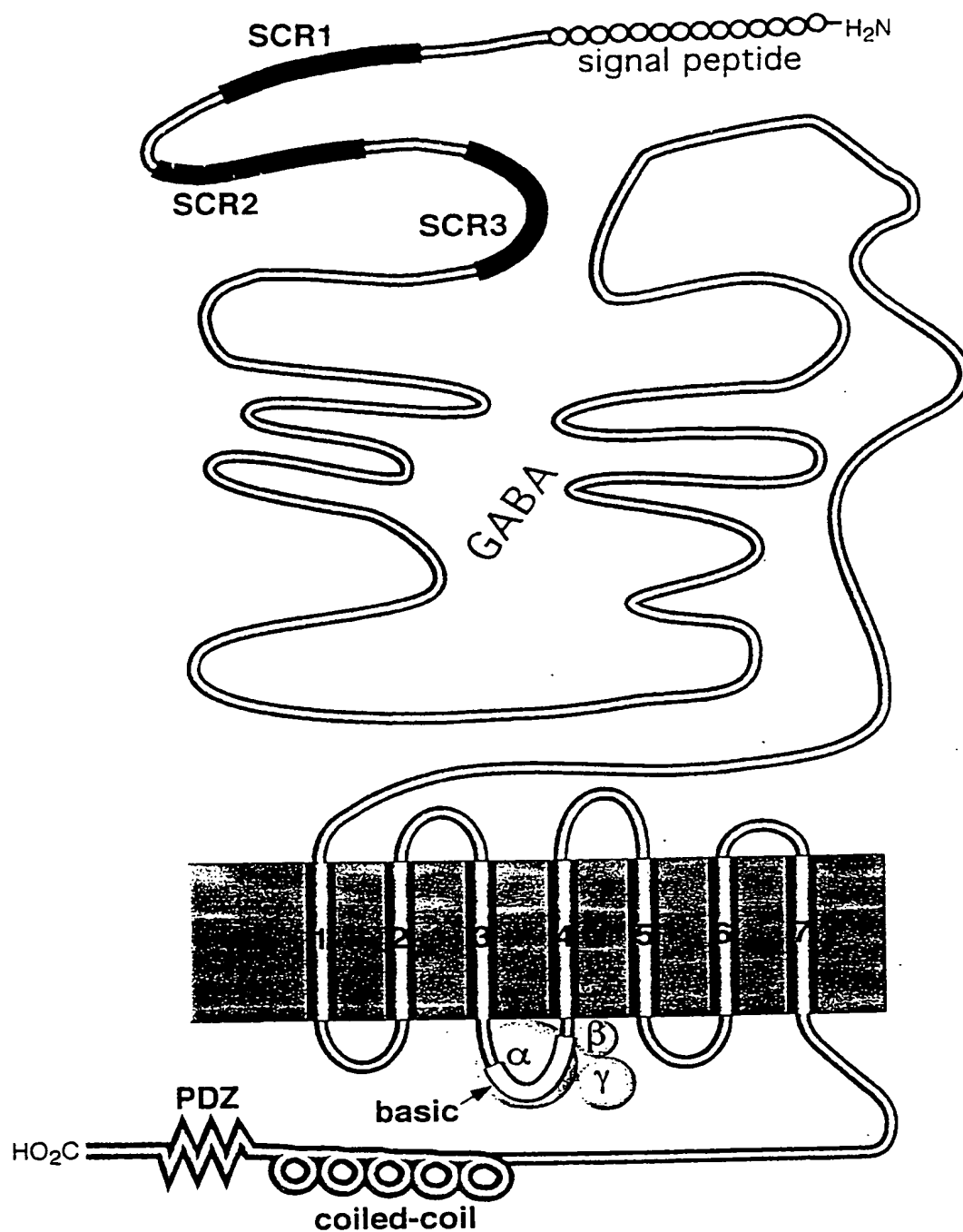


FIGURE 27

mGABAb1a .KMR...RLITRGEW...QSETQDQTMKTGSS.TNNNEEEKSRL...LEKE: 39  
 hGABAb1a PKMR...RLITRGEW...QSEAQDQTMKTGSS.TNNNEEEKSRL...LEKE: 40  
 hGABAb2 ITLR TNPDAATQNRRFQFTQNKKEDESKTSTSVTSVQASTSRLEGLQSE: 50  
 mGABAb1a NR...ELEKIIAE...KEERVSE...: 56  
 hGABAb1a NR...ELEKIIAE...KEERVSE...: 57  
 hGABAb2 NHRLRMKITELEDKDLEEVMTMQLQDTPEKTTYIKQNHYQELNDILNLGNFT:100  
 mGABAb1a .LRHQLOSRQQL.RSRRHPP.TPPDPSSGGLPRGPPSEPPDRLS: 95  
 hGABAb1a .LRHQLOSRQQL.RSRRHPP.TPPEPSGGLPRGPPSEPPDRLS: 96  
 hGABAb2 ESTDGGKAILKKNHLDQNPQLQWNTTEPSRTCKDPIEDI.NSPEHIQRRLS:149  
 PDZ  
 mGABAb1a .CDGSRV...HLL.YK...:106  
 hGABAb1a .CDGSRV...HLL.YK...:107  
 hGABAb2 LQLPILHHAYLPSIGGVDA[CV]SPCVSP[ASPRHRH]VPPSFRVMVSGL:197

← coiled-coil domain  
 ←

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/02361

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 14/705; C12N 5/10, 15/09, 15/11, 15/12, 15/62; G01N 33/566

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 23.5; 530/350; 435/6, 7.1, 7.2, 69.1, 320.1, 325, 252.3, 254.11

Documentation searched other than minimum: documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

GenBank, APS, Medline, Biosis, Caplus

search terms: HG20, gaba? receptor, t mcdonald, t bonnert, gaba?, gamma?

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GenBank Database, National Library of Medicine, Bethesda, Maryland USA, HILLIER et al., Accession Number H14151, ym62d04.rl Homo sapiens cDNA clone 163495 5', 10 July 1995.	3
X	GenBank Database, Bethesda, National Library of Medicine, Maryland USA, GENEXPRESS, Accession Number Z43654, H. Sapiens partial cDNA sequence; clone c-1hh04, 21 September 1995.	3
X	GenBank Database, National Library of Medicine, Bethesda, Maryland USA, ADAMS et al., Accession Number T07621, EST05511 Homo sapiens cDNA clone HFBEL81, 30 June 1993.	3

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 01 JUNE 1999	Date of mailing of the international search report 02 JUL 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CLAIRE KAUFMAN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)\*

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/02361

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ----- A,P	WO 97/46675 A1 (NOVARTIS) 11 December 1997, especially claims 1 and 3.	3 ----- 1, 2, 4, 5, 7-9, 14, 16, 18, 19

Form PCT/ISA/210 (continuation of second sheet)(July 1992)★

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/02361

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.: 6  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
  
THERE IS NO RECITATION OF WHAT IS INCLUDED IN THE GROUP FROM WHICH THE ENCODED PROTEIN IS SELECTED.
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-5, 7-9, 14, 16, 18, 19

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/02361

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

536/23.1, 23.5; 530/350; 435/6, 7.1, 7.2, 69.1, 320.1, 325, 252.3, 254.11

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-5, 7-9, 14, 16, 18, 19, drawn to HG20 DNA, vector, host cell, protein, heterodimer comprising the protein, method of detecting binding to GABAB receptors, method of producing functional GABAB receptors, and method of expression an amino-terminal truncated HG20.

Group II, claim(s) 10-11, drawn to a polypeptide consisting of a coiled-coil domain.

Group III, claim(s) 12-13, drawn to DNA encoding a GABABR1a polypeptide and GABABR1a protein.

Group IV, claim(s) 15, drawn to method of identifying agonists and antagonists of HG20.

Group V, claim(s) 17, drawn to antibody that binds HG20.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Pursuant to 37 C.F.R. 1.475(d), this Authority considers that the main invention in the instant application comprises the first-recited product, DNA encoding HG20, and the first-recited method of using that product, namely the method of detecting binding to GABAB receptors. Note that there is no method of making the polynucleotide. Also included in the first group is the encoded protein and the process of producing the encoded protein, in addition to a heterodimer, vector and host cell. Further, pursuant to 37 C.F.R. 1.475(b)-(d), the ISA/US considers that the materially and functionally dissimilar product of groups II and V and the additional methods of groups III-IV do not correspond to the main invention. This Authority therefore considers that the several inventions do not share a special technical feature within the meaning of PCT Rule 13.2 and thus do not relate to a single general inventive concept within the meaning of PCT Rule 13.1.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup>:</b> <b>C07K 14/705, C12N 5/10, 15/09, 15/11, 15/12, 15/62, G01N 33/566</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/40114</b> <b>(43) International Publication Date:</b> 12 August 1999 (12.08.99)
<b>(21) International Application Number:</b> PCT/US99/02361 <b>(22) International Filing Date:</b> 3 February 1999 (03.02.99)  <b>(30) Priority Data:</b> 60/073,767      5 February 1998 (05.02.98)      US  <b>(71) Applicants (for all designated States except US):</b> MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). MERCK FROSST CANADA & CO. [CA/CA]; 16711 Trans-Canada Highway, Kirkland, Quebec H9H 3L1 (CA). UNIVERSITY OF TEXAS HEALTH SCIENCE CENTER AT SAN ANTONIO [US/US]; 7703 Floyd Curl Drive, San Antonio, TX 78284 (US). NATIONAL INSTITUTES OF HEALTH [US/US]; 36 Convent Drive, Bethesda, MD 20892-4094 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> LIU, Quingyun [CN/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). McDONALD, Terrence [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). BONNERT, Timothy, P. [GB/GB]; Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR (GB). NG, Gordon, Yiu, Kon [CA/CA]; 16711 Trans-Canada Highway, Kirkland, Quebec H9H 3L1 (CA). KOLAKOWSKI, Lee, F., Jr. [US/US]; 7703 Floyd	<b>(74) Common Representative:</b> MERCK & CO., INC.; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).  <b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> NOVEL GABA <sub>B</sub> RECEPTOR DNA SEQUENCES		
<b>(57) Abstract</b>  DNA encoding a novel human GABA <sub>B</sub> receptor subunit, HG20, as well as the protein encoded by the DNA, is provided. Also provided is DNA encoding a novel murine GABA <sub>B</sub> receptor subunit, GABA <sub>B</sub> R1a, as well as the protein encoded by the DNA. Heterodimers of HG20 protein and GABA <sub>B</sub> R1a protein that form a functional GABA <sub>B</sub> receptor are disclosed. Methods of identifying agonists and antagonists of the GABA <sub>B</sub> receptor are also provided.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						



TITLE OF THE INVENTION  
NOVEL GABA<sub>B</sub> RECEPTOR DNA SEQUENCES

CROSS-REFERENCE TO RELATED APPLICATIONS

5 Not applicable.

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not applicable.

10 REFERENCE TO MICROFICHE APPENDIX

Not applicable.

FIELD OF THE INVENTION

15 The present invention is directed to a novel human DNA sequence encoding HG20, a subunit of the GABA<sub>B</sub> receptor, the protein encoded by the DNA, and uses thereof. The present invention also is directed to the murine GABA<sub>B</sub>R1a subunit of the GABA<sub>B</sub> receptor as well as to methods of combining an HG20 subunit with a GABA<sub>B</sub>R1a subunit to form a GABA<sub>B</sub> receptor having functional activity.

20

BACKGROUND OF THE INVENTION

Amino acids such as glutamic acid,  $\gamma$ -amino-butyric acid (GABA), and glycine are neurotransmitters that bind to specific receptors in the vertebrate nervous system and mediate synaptic transmission. Of  
25 these amino acids, GABA is the most widely distributed amino acid inhibitory neurotransmitter in the vertebrate central nervous system. The biological activities of GABA are mediated by three types of GABA receptors: ionotropic GABA<sub>A</sub> receptors, metabotropic GABA<sub>B</sub> receptors, and ionotropic GABA<sub>C</sub> receptors. Each type of receptor has its own  
30 characteristic molecular structure, pattern of gene expression, agonist and antagonist mediated pharmacological effects, and spectrum of physiological activities.

GABA<sub>A</sub> receptors mediate fast synaptic inhibition. They are heterooligomeric proteins (most likely pentamers) containing  $\alpha$ ,  $\beta$ ,  $\gamma$ , and

perhaps  $\delta$ , subunits that function as ligand-gated  $\text{Cl}^-$  channels and have binding sites for benzodiazepines, barbiturates, and neuroactive steroids. Bicuculline is a widely used antagonist of  $\text{GABA}_A$  receptors. Bicuculline is selective for  $\text{GABA}_A$  receptors in that it has no effect on  $\text{GABA}_B$  or

5  $\text{GABA}_C$  receptors. The expression of  $\text{GABA}_A$  receptors has been observed in a variety of brain structures (see. *e.g.*, McKernan & Whiting, 1996, Trends Neurosci. 16:139-143; Sequier et al., 1988, Proc. Natl. Acad. Sci. USA 85:7815-7819).

$\text{GABA}_C$  receptors are ligand-gated  $\text{Cl}^-$  channels found in the

10 vertebrate retina. They can be distinguished from  $\text{GABA}_A$  and  $\text{GABA}_B$  receptors in that they are insensitive to the  $\text{GABA}_A$  receptor antagonist bicuculline and the  $\text{GABA}_B$  receptor agonist (-)baclofen but are selectively activated by *cis*-4-aminocrotonic acid.  $\text{GABA}_C$  receptors are composed of homooligomers of a category of GABA receptor subunits known as " $\rho$ "

15 subunits, the best-studied of which are  $\rho 1$  and  $\rho 2$ .  $\rho 1$  and  $\rho 2$  share 74% amino acid sequence identity but are only about 30-38% identical in amino acid sequence when compared to  $\text{GABA}_A$  receptor subunits. For a review of  $\text{GABA}_C$  receptors, see Bormann & Feigenspan, 1995, Trends Neurosci. 18:515-518.

$\text{GABA}_B$  receptors play a role in the mediation of late

20 inhibitory postsynaptic potentials (IPSPs).  $\text{GABA}_B$  receptors belong to the superfamily of seven transmembrane-spanning G-protein coupled receptors that are coupled through G-proteins to neuronal  $\text{K}^+$  or  $\text{Ca}^{++}$  channels.  $\text{GABA}_B$  receptors are coupled through G-proteins to neuronal

25  $\text{K}^+$  or  $\text{Ca}^{++}$  channels, and receptor activation increases  $\text{K}^+$  or decreases  $\text{Ca}^{++}$  conductance and also inhibits or potentiates stimulated adenylyl cyclase activity. The expression of  $\text{GABA}_B$  receptors is widely distributed in the mammalian brain (*e.g.*, frontal cortex, cerebellar molecular layer, interpeduncular nucleus) and has been observed in many peripheral

30 organs as well.

A large number of pharmacological activities have been attributed to  $\text{GABA}_B$  receptor activation, *e.g.*, analgesia; hypothermia; catatonia; hypotension; reduction of memory consolidation and retention; and stimulation of insulin, growth hormone, and glucagon release (see

Bowery, 1989, Trends Pharmacol. Sci. 10:401-407, for a review.) It is well accepted that GABA<sub>B</sub> receptor agonists and antagonists are pharmacologically useful. For example, the GABA<sub>B</sub> receptor agonist (-)baclofen, a structural analog of GABA, is a clinically effective muscle relaxant (Bowery & Pratt, 1992, Arzneim.-Forsch./Drug Res. 42:215-223). (-)baclofen, as part of a racemic mixture with (+)baclofen, has been sold in the United States as a muscle relaxant under the name LIORESAL® since 1972.

GABA<sub>B</sub> receptors represent a large family of related proteins, new family members of which are still being discovered. For example, Kaupmann et al., 1997, Nature 386:239-246 (Kaupmann) reported the cloning and expression of two members of the rat GABA<sub>B</sub> receptor family, GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b. A variety of experiments using known agonists and antagonists of GABA<sub>B</sub> receptors seemed to indicate that GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b represented rat GABA<sub>B</sub> receptors. This conclusion was based primarily on the ability of GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b to bind agonists and antagonist of GABA<sub>B</sub> receptors with the expected rank order, based upon studies of rat cerebral cortex GABA<sub>B</sub> receptors. However, there were data that did not fit the theory that Kaupmann had cloned the pharmacologically and functionally active GABA<sub>B</sub> receptor. For example, Kaupmann noted that agonists had significantly lower binding affinity to recombinant GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b as opposed to native GABA<sub>B</sub> receptors. Also, Couve et al., 1998, J. Biol. Chem. 273:26361-26367 showed that recombinantly expressed GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b failed to target correctly to the plasma membrane and failed to give rise to functional GABA<sub>B</sub> receptors when expressed in a variety of cell types.

Examination of the amino acid and gene sequence of GABA<sub>B</sub>R1a led Kaupmann to propose a structure for GABA<sub>B</sub>R1a similar to that of the metabotropic glutamate receptor gene family. The metabotropic glutamate receptor family comprises eight glutamate binding receptors and five calcium sensing receptors which exhibit a signal peptide sequence followed by a large N-terminal domain believed to represent the ligand binding pocket that precedes seven transmembrane

spanning domains. The hallmark seven transmembrane spanning domains are typical of G-protein coupled receptors (GPCRs), although metabotropic glutamate receptors and GABA<sub>B</sub>R1a are considerably larger than most GPCRs and contain a signal peptide sequence. No significant amino acid sequence similarities were found between GABA<sub>B</sub>R1a and GABA<sub>A</sub> receptors, GABA<sub>C</sub> receptors, or other typical GPCRs.

Despite work such as that of Kaupmann, pharmacological and physiological evidence indicates that a large number of amino acid binding GABA<sub>B</sub> receptors remain to be cloned and expressed in recombinant systems where agonists and antagonists can be efficiently identified. In particular, it would be extremely valuable to be able to recombinantly express GABA<sub>B</sub> receptors in such a manner that not only pharmacologically relevant ligand binding properties would be exhibited by the recombinant receptors, but also such that the recombinant receptors would show proper functional activity.

#### SUMMARY OF THE INVENTION

The present invention is directed to a novel human DNA that encodes a GABA<sub>B</sub> receptor subunit, HG20. The DNA encoding HG20 is substantially free from other nucleic acids and has the nucleotide sequence shown in SEQ.ID.NO.:1. Also provided is an HG20 protein encoded by the novel DNA sequence. The HG20 protein is substantially free from other proteins and has the amino acid sequence shown in SEQ.ID.NO.:2. Methods of expressing HG20 in recombinant systems and of identifying agonists and antagonists of HG20 are provided.

The present invention is also directed to a novel murine DNA that encodes a GABA<sub>B</sub> receptor subunit, GABA<sub>B</sub>R1a. The DNA encoding GABA<sub>B</sub>R1a is substantially free from other nucleic acids and has the nucleotide sequence shown in SEQ.ID.NO.:19. Also provided is a GABA<sub>B</sub>R1a protein encoded by the novel DNA sequence. The GABA<sub>B</sub>R1a protein is substantially free from other proteins and has the amino acid sequence shown in SEQ.ID.NO.:20. Methods of expressing GABA<sub>B</sub>R1a in recombinant systems and of identifying agonists and antagonists of HG20 are provided.

Also provided by the present invention are methods of co-expressing HG20 and GABA<sub>B</sub>R1a in the same cells. Such co-expression results in the production of a GABA<sub>B</sub> receptor that exhibits expected functional properties of GABA<sub>B</sub> receptors as well as expected ligand binding properties. Recombinant cells co-expressing HG20 and GABA<sub>B</sub>R1a are provided as well as methods of utilizing such recombinant cells to identify agonists and antagonists of GABA<sub>B</sub> receptors.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-B shows the complete cDNA sequence of HG20 (SEQ.ID.NO.:1).

Figure 2 shows the complete amino acid sequence of HG20 (SEQ.ID.NO.:2).

Figure 3A-B shows predicted signal peptide cleavage sites of HG20. All sequences shown are portions of SEQ.ID.NO.:2.

Figure 4 shows *in situ* analysis of the expression of HG20 RNA in squirrel monkey brain.

Figure 5A shows *in vitro* coupled transcription/translation of a chimeric FLAG epitope-HG20 (amino acids 52-941) protein.

Figure 5B shows the expression in COS-7 cells and melanophores of a chimeric FLAG epitope-HG20 (amino acids 52-941) protein.

Figure 6 shows a comparison of the amino acid sequences of a portion of the N-terminus of HG20 protein and the ligand binding domain of the *Pseudomonas aeruginosa* amino acid binding protein LIVAT-BP (Swiss Protein database accession number P21175). The upper sequence shown is from HG20 and corresponds to amino acids 63-259 of SEQ.ID.NO.:2. The lower sequence shown is from *Pseudomonas aeruginosa* LIVAT-BP and is SEQ.ID.NO.:16.

Figure 7 shows expression in mammalian cells of a chimeric HG20 protein.

Figure 8 shows a comparison of the amino acid sequences of HG20 and GABA<sub>B</sub>R1b. The HG20 sequence is SEQ.ID.NO.:2. The GABA<sub>B</sub>R1b sequence is SEQ.ID.NO.:17.

Figure 9 shows the expression of recombinant GABA<sub>B</sub>R1a and HG20 in COS-7 cells. Lanes 1 and 2 show [<sup>125</sup>I]CGP71872 photolabeling of recombinant murine GABA<sub>B</sub>R1a monomer and dimer in the presence (+) and absence (-) of 1 μM unlabeled CGP71872. Lanes 3 and 4 show that GABA<sub>B</sub>R1a antibodies 1713.1-1713.2 confirmed (+) expression of recombinantly expressed murine GABA<sub>B</sub>R1a (referred to as mgb1a here) and absence (-) in pcDNA3.1 mock transfected cells. Lanes 5 and 6 show [<sup>125</sup>I]CGP71872 photolabeling of human FLAG-HG20 in the presence (+) and absence (-) of 1 μM unlabeled CGP71872. Lanes 7 and 8 show that an anti-FLAG antibody confirmed (+) the expression of FLAG-HG20 (referred to as FLAG-gb2 here) and its absence (-) in pcDNA3.1 mock transfected cells. Experimental details were as in Examples 7-9 and 20 except that COS-7 rather than COS-1 cells were used.

Figure 10 shows co-localization of mRNA for HG20 and GABA<sub>B</sub>R1a by *in situ* hybridization histochemistry in rat parietal cortex. Adjacent coronal sections of rat brain showing parietal cortex hybridized with radiolabelled GABA<sub>B</sub>R1a (A) and HG20 (B) probes. Rat GABA<sub>B</sub>R1a and HG20 probes were labelled using <sup>35</sup>S-UTP (A, B, and D), and autoradiograms were developed after 4 weeks. For co-localization studies, the rat GABA<sub>B</sub>R1a probe was digoxigenin labelled and developed using anti-digoxigenin HRP, the TSA amplification method and biotinyl tyramide followed by streptavidin-conjugated CY3 (C). (D) shows autoradiography of the same field as in (C), denoting hybridization to HG20 mRNA. (E) is an overlay of images (C) and (D). Arrows denote some of the double-labelled cells. Scale bar = 0.5 mm in (A) and (B); scale bar = 50 μm in (C-E).

Figure 11 shows functional complementation following co-expression of GABA<sub>B</sub>R1a and HG20 in *Xenopus* melanophores. GABA mediated a dose-dependent aggregation response in melanophores co-expressing murine GABA<sub>B</sub>R1a and FLAG-HG20 (■) that could be blocked with 100 nM (▼) and 1 μM CGP71872 (▲). The response of GABA on mock-transfected cells is shown (●) as well as a control cannabinoid receptor subtype 2 response to HU210 ligand (inset). This experiment is representative of n=4.

Figure 12 shows GABA<sub>B</sub> receptor modulation of forskolin-stimulated cAMP synthesis in HEK293 cells. HEK293 cells stably expressing HG20 (hgb2-42) or GABA<sub>B</sub>R1a (rgb1a-50) were transiently transfected with GABA<sub>B</sub>R1a and HG20 expression plasmids to examine the effect of receptor co-expression on modulation of cAMP synthesis. All transfected cells were tested with 300  $\mu$ M baclofen or GABA (with 100  $\mu$ M AOAA and 100  $\mu$ M nipecotic acid) in the absence of forskolin and 30  $\mu$ M baclofen or GABA in the presence of 10  $\mu$ M forskolin. Wild-type HEK293 cells were tested with 250  $\mu$ M baclofen or 250  $\mu$ M GABA in the presence of 10  $\mu$ M forskolin. Data are presented as the percent of total cAMP synthesized in the presence of forskolin only. The data presented are from single representative experiments that have been replicated twice. Fsk, forskolin; B, baclofen; G, GABA with AOAA and nipecotic acid. The two right-most set of bar graphs (labeled "B + Fsk" and "G + Fsk") show that in cells expressing both GABA<sub>B</sub>R1a and HG20 (rgb1a-50/hgb2 cells ( $\square$ )) and hgb2-42/rgb1a cells ( $\blacksquare$ )), baclofen and GABA were able to mediate significant reductions in cAMP levels.

Figure 13 shows that co-expression of GABA<sub>B</sub>R1a and HG20 permits inwardly rectifying potassium channel (GIRK or Kir) activation in *Xenopus* oocytes. (A) Representative current families of Kir 3.1/3.2. Currents were evoked by 500 msec voltage commands from a holding potential of -10 mV, delivered in 20 mV increments from -140 to 60 mV. (B) In a protocol designed to measure the effects of various receptors on Kir currents, oocytes were held at -80 mV (a potential where significant inward current is measured). Expression of GABA<sub>B</sub>R1a or HG20 alone (with or without G $\alpha$ 1) resulted in no modulation of current after GABA treatment. Co-expression of GABA<sub>B</sub>R1a and FLAG-HG20 receptors followed by treatment with 100  $\mu$ M GABA resulted in stimulation of Kir 3.1/3.2. Shown are representative traces from at least three independent experiments under each condition.

Figure 14 shows immunoblotting of murine GABA<sub>B</sub>R1a and FLAG-HG20 transiently expressed in COS-7 cells. Digitonin-solubilized and anti-FLAG antibody immunoprecipitated membrane proteins were immunoblotted following SDS-PAGE with GABA<sub>B</sub>R1a antibodies 1713.1-

1713.2. The conditions are as follows: mock pcDNA3.1 vector transfected cells (lane 1), FLAG-HG20 expressing cells (lane 2), murine GABABR1a expressing cells (lane 3), and cells coexpressing murine GABABR1a and FLAG-HG20 (lane 4). The immunoreactive band corresponding to the GABABR1a /HG20 heterodimer as well as a band corresponding to the predicted GABABR1a monomer are denoted by arrows.

Figure 15 shows the complete cDNA sequence of murine GABABR1a (SEQ.ID.NO.:19). The sequence shown has been deposited in GenBank (accession number AF114168).

Figure 16 shows the complete amino acid sequence of murine GABABR1a (SEQ.ID.NO.:20). The sequence shown has been deposited in GenBank (accession number AF114168).

Figure 17A-B shows the results of experiments with N- and C-terminal fragments of murine GABABR1a. Figure 17A shows the results of coupled *in vitro* transcription/translation reactions; lane 1 = blank; lane 2 = full-length GABABR1a; lane 3 = N-terminal fragment of GABABR1a; lane 4 = C-terminal fragment of GABABR1a. Figure 17B shows the results of [<sup>125</sup>I]CGP71872 photoaffinity labeling; lane 1 = N-terminal fragment of GABABR1a; lane 2 = N-terminal fragment of GABABR1a in the presence of GABA; lane 3 = C-terminal fragment of GABABR1a; lane 4 = C-terminal fragment of GABABR1a in the presence of GABA.

Figure 18A-B shows the amino acid sequence (Figure 18A) (SEQ.ID.NO.:21) and nucleotide sequence (Figure 18B) (SEQ.ID.NO.:22) (GenBank accession number AJ012185) of a human GABABR1a.

Figure 19A-B shows the nucleotide sequence (SEQ.ID.NO.:23) (GenBank accession number Y11044) of a human GABABR1a.

Figure 20 shows a framework map of chromosome 9. The locations of the HG20 gene (referred to as "GPR 51"), markers, and the HSN-1 locus are indicated.

Figure 21 shows a hydropathy plot for murine GABABR1a.

Figure 22 shows a family tree of genes related to HG20. Abbreviations are as follows: hGB1a = human GABABR1a; mGB1a =



mouse GABA<sub>B</sub>R1a; rGB1a = rat GABA<sub>B</sub>R1a; hGB1b = human GABA<sub>B</sub>R1b; rGB1b = rat GABA<sub>B</sub>R1b; ceGB1b = a *C. elegans* gene related to mammalian GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b; hGB2 = human HG20; ceGB2 = a *C. elegans* gene related to human HG20; MGRDROME = a  
 5 metabotropic glutamate receptor from *Drosophila melanogaster*; MGR2 HUMAN = human metabotropic glutamate receptor 2; MGR3 HUMAN = human metabotropic glutamate receptor 3; MGR6 HUMAN = human metabotropic glutamate receptor 6; MGR4 HUMAN = human metabotropic glutamate receptor 4; MGR7 HUMAN = human  
 10 metabotropic glutamate receptor 7; MGR8 HUMAN = human metabotropic glutamate receptor 8; MGR1 HUMAN = human metabotropic glutamate receptor 1; MGR5 HUMAN = human metabotropic glutamate receptor 5.

Figure 23 shows the coiled-coil domains in the C-termini of  
 15 human GABA<sub>B</sub>R1a and HG20. The upper sequence is from human GABA<sub>B</sub>R1a and is positions 886-949 of SEQ.ID.NO.:21. The lower sequence is from HG20 and is positions 756-829 of SEQ.ID.NO.:2.

Figure 24 shows a comparison of the amino acid sequences of human GABA<sub>B</sub>R1a (referred to as "Human GABA-B1aR,"  
 20 SEQ.ID.NO.:21); the proteins encoded by two genes from *C. elegans* (*C. elegans* GABA-B1 = SEQ.ID.NO.:42 and *C. elegans* GABA-B2 = SEQ.ID.NO.:43); and HG20) (referred to as "Human GABA-B2," (SEQ.ID.NO.:2). The *C. elegans* genes have been predicted from *C. elegans* DNA sequence alone. ZK180 accession number: U58748 is predicted to be  
 25 GABA-B2 and Y41G9. Contig99 and Y76F7.Contig73 were obtained from the Sanger *C. elegans* genomic sequence database and are predicted to be GABA-B1.

Figure 25A-D shows co-immunoprecipitation of the murine GABA<sub>B</sub>R1a and FLAG-HG20 receptor subunits and immunoblotting  
 30 using reciprocal receptor subunit antibodies. Murine GABA<sub>B</sub>R1a and FLAG-HG20 receptors were expressed individually or co-expressed in COS-7 cells. Figure 25A shows the results of immunoblotting using an anti-murine GABA<sub>B</sub>R1a antibody. Immunoblot of the solubilized membranes using murine GABA<sub>B</sub>R1a antibodies 1713.1-1713.2 shows

selective expression of murine GABA<sub>B</sub>R1a in murine GABA<sub>B</sub>R1a alone expressing cells (lane 3) and murine GABA<sub>B</sub>R1a /FLAG-HG20 co-expressing cells (lane 4), but not in mock transfected and FLAG-HG20 alone expressing cells (lanes 1 and 2). Staining of GABA<sub>B</sub>R1a subunits in

5 co-expressing cells is more intense compared to cells expressing the GABA<sub>B</sub>R1a subunit alone, suggesting that HG20 subunits facilitate GABA<sub>B</sub>R1a expression. Figure 25B shows the results of immunoblotting using an anti-FLAG-HG20 antibody. Immunoblotting of the solubilized membranes using the anti-FLAG-HG20 antibody shows selective

10 expression of FLAG-HG20 subunits in FLAG-HG20 alone expressing cells (lane 6) and murine GABA<sub>B</sub>R1a /FLAG-HG20 co-expressing cells (lane 8), but not in mock transfected and murine GABA<sub>B</sub>R1a alone expressing cells (lanes 5 and 7). Staining of HG20 subunits in co-expressing cells is more intense compared to cells expressing the HG20 subunit alone, suggesting

15 that GABA<sub>B</sub>R1a subunits facilitate HG20 expression. Figure 25C shows the results of immunoprecipitation with an anti-FLAG-HG20 antibody followed by immunoblotting with an anti-murine GABA<sub>B</sub>R1a antibody. GABA<sub>B</sub>R1a /HG20 heterodimers are observed only in murine GABA<sub>B</sub>R1a /FLAG-HG20 co-expressing cells due to the fact that the GABA<sub>B</sub>R1a

20 subunit was co-immunoprecipitated with the FLAG-HG20 subunit using the FLAG antibody and detected with GABA<sub>B</sub>R1a antibodies (lane 12). GABA<sub>B</sub>R1a subunits are not detected in mock-transfected cells and cells expressing GABA<sub>B</sub>R1a alone or FLAG-HG20 (lanes 9-11). Figure 25D shows the results of immunoprecipitation with an anti-murine

25 GABA<sub>B</sub>R1a antibody followed by immunoblotting with an anti-FLAG-HG20 antibody. GABA<sub>B</sub>R1a /HG20 heterodimers are observed only in murine GABA<sub>B</sub>R1a /FLAG-HG20 co-expressing cells due to the fact that the FLAG-HG20 subunit was co-immunoprecipitated using the GABA<sub>B</sub>R1a antibodies and detected with FLAG antibody (lane 16). No

30 FLAG-HG20 subunits are detected in mock-transfected cells or cells expressing murine GABA<sub>B</sub>R1a alone or FLAG-HG20 (lanes 13-15). The immunoblots shown are from 1-3 independent experiments.

Figure 26A-B shows some of the motifs in the N-termini of GABA<sub>B</sub> receptor subunits and related genes. Figure 26A shows an

alignment of murine GABA<sub>B</sub>R1a (mGABA<sub>B</sub>1a; a portion of  
SEQ.ID.NO.:20), human GABA<sub>B</sub>R1a (hGABA<sub>B</sub>1a; a portion of  
SEQ.ID.NO.:21), HG20 (hGABA<sub>B</sub>2; a portion of SEQ.ID.NO.:2),  
metabotropic glutamate receptor 1 (mGluR1; SEQ.ID.NO.:44), and two *E.*  
5 *coli* proteins (LivK (SEQ.ID.NO.:45) and LivBP (SEQ.ID.NO.:46)).

Figure 26B is a schematic drawing showing the location of the various  
motifs in murine GABA<sub>B</sub>R1a that are expected to be involved in  
heterodimer formation of GABA<sub>B</sub>R1a with HG20.

Figure 27 shows an expanded view of the coiled-coil region of  
10 homology between HG20 (hGABA<sub>B</sub>2; shown is a portion of SEQ.ID.NO.:2)  
and murine GABA<sub>B</sub>R1a (mGABA<sub>B</sub>1a; a portion of SEQ.ID.NO.:20). Also  
shown is the corresponding region of human GABA<sub>B</sub>R1a (hGABA<sub>B</sub>1a; a  
portion of SEQ.ID.NO.:21).

## 15 DETAILED DESCRIPTION OF THE INVENTION

For the purposes of this invention:

"Substantially free from other proteins" means at least 90%,  
preferably 95%, more preferably 99%, and even more preferably 99.9%,  
free of other proteins. Thus, for example, an HG20 protein preparation  
20 that is substantially free from other proteins will contain, as a percent of  
its total protein, no more than 10%, preferably no more than 5%, more  
preferably no more than 1%, and even more preferably no more than 0.1%,  
of non-HG20 proteins. Whether a given HG20 protein preparation is  
substantially free from other proteins can be determined by such  
25 conventional techniques of assessing protein purity as, *e.g.*, sodium  
dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) combined  
with appropriate detection methods, *e.g.*, silver staining or  
immunoblotting.

"Substantially free from other nucleic acids" means at least  
30 90%, preferably 95%, more preferably 99%, and even more preferably  
99.9%, free of other nucleic acids. Thus, for example, an HG20 DNA  
preparation that is substantially free from other nucleic acids will contain,  
as a percent of its total nucleic acid, no more than 10%, preferably no  
more than 5%, more preferably no more than 1%, and even more

preferably no more than 0.1%, of non-HG20 nucleic acids. Whether a given HG20 DNA preparation is substantially free from other nucleic acids can be determined by such conventional techniques of assessing nucleic acid purity as, *e.g.*, agarose gel electrophoresis combined with  
5 appropriate staining methods, *e.g.*, ethidium bromide staining, or by sequencing.

An HG20 polypeptide has "substantially the same biological activity" as native HG20 (*i.e.*, SEQ.ID.NO.:2) if that polypeptide has a  $K_d$  for a ligand that is no more than 5-fold greater than the  $K_d$  of native  
10 HG20 for the same ligand. An HG20 polypeptide also has "substantially the same biological activity" as HG20 if that polypeptide can form heterodimers with either a GABABR1a or GABABR1b polypeptide, thus forming a functional GABAB receptor.

"Functional GABAB receptor" refers to a heterodimer of  
15 HG20 and either GABABR1a or GABABR1b where the heterodimer displays a functional response when exposed to GABA agonists. Examples of functional responses are: pigment aggregation in *Xenopus* melanophores, modulation of cAMP levels, coupling to inwardly rectifying potassium channels, mediation of late inhibitory postsynaptic potentials  
20 in neurons, increase in potassium conductance, and decrease in calcium conductance. One skilled in the art would be familiar with a variety of methods of measuring the functional responses of G-protein coupled receptors such as the GABAB receptor (see, *e.g.*, Lerner, 1994, Trends Neurosci. 17:142-146 [changes in pigment distribution in melanophore  
25 cells]; Yokomizo et al., 1997, Nature 387:620-624 [changes in cAMP or calcium concentration; chemotaxis]; Howard et al., 1996, Science 273:974-977 [changes in membrane currents in *Xenopus* oocytes]; McKee et al., 1997, Mol. Endocrinol. 11:415-423 [changes in calcium concentration measured using the aequorin assay]; Offermanns & Simon, 1995, J. Biol.  
30 Chem. 270:15175, 15180 [changes in inositol phosphate levels]). Depending upon the cells in which heterodimers of HG20 and either GABABR1a or GABABR1b are expressed, and thus the G-proteins with which the heterodimers are coupled, certain of such methods may be appropriate for measuring the functional responses of such heterodimers.

It is well with the competence of one skilled in the art to select the appropriate method of measuring functional responses for a given experimental system.

5 A GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b polypeptide has "substantially the same biological activity" as a native GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b polypeptide if that polypeptide has a  $K_d$  for an amino acid, amino acid analogue, GABA<sub>B</sub> receptor agonist, or GABA<sub>B</sub> receptor antagonist such as CGP71872, GABA, saclofen, (-)baclofen, or (L)-glutamic acid that is no more than 5-fold greater than the  $K_d$  of a native GABA<sub>B</sub>R1a or  
10 GABA<sub>B</sub>R1b polypeptide for the same amino acid, amino acid analogue, GABA<sub>B</sub> receptor agonist, or GABA<sub>B</sub> receptor antagonist. A GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b polypeptide also has "substantially the same biological activity" as a native GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b polypeptide if that polypeptide can form heterodimers with an HG20 polypeptide, thus  
15 forming a functional GABA<sub>B</sub> receptor. Native GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b polypeptides include the murine GABA<sub>B</sub>R1a sequence shown as SEQ.ID.NO.:20; the rat GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b polypeptides disclosed in Kaupmann et al., 1997, Nature 386:239-246; the human GABA<sub>B</sub>R1a sequence disclosed in GenBank accession number AJ012185  
20 (SEQ.ID.NO.:21); and the protein encoded by the DNA sequence disclosed in GenBank accession number Y11044 (SEQ.ID.NO.:23).

A "conservative amino acid substitution" refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are:  
25 substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (e.g., arginine for lysine; glutamic acid for aspartic acid).

The present invention relates to the identification and  
30 cloning of HG20, a novel G-protein coupled receptor-like protein that represents a subunit for the GABA<sub>B</sub> receptor. The present invention provides DNA encoding HG20 that is substantially free from other nucleic acids. The present invention also provides recombinant DNA molecules encoding HG20 as well as isolated DNA molecules encoding HG20.

Following the cloning of HG20 by the present inventors, a sequence highly similar to the sequence of HG20 was deposited in GenBank by Clark et al. (GenBank accession number AF056085), by White et al. (GenBank accession number AJ012188), and by Borowsky et al. (GenBank accession number AF074483). Two ESTs (GenBank accession number T07621, deposited June 30, 1993, and GenBank accession number Z43654, deposited September 21, 1995) each contain partial sequences of HG20 cDNA.

The present invention provides a DNA molecule substantially free from other nucleic acids comprising the nucleotide sequence shown in Figure 1 as SEQ.ID.NO.:1. Analysis of SEQ.ID.NO.:1 revealed that it contains a long open reading frame at positions 293-3,115. Thus, the present invention also provides a DNA molecule substantially free from other nucleic acids comprising the nucleotide sequence of positions 293-3,115 of SEQ.ID.NO.:1. The present invention also provides an isolated DNA molecule comprising the nucleotide sequence of positions 293-3,115 of SEQ.ID.NO.:1.

Sequence analysis of the open reading frame of the HG20 DNA revealed that it encodes a protein of 941 amino acids with a calculated molecular weight of 104 kd and a predicted signal peptide. The predicted amino acid sequence of HG20 is 36% identical to the metabotropic GABA receptor-like sequence GABABR1a described in Kaupmann (see above) throughout the entire sequence, and thus HG20 most likely represents a novel metabotropic GABA receptor or receptor subunit. *In situ* hybridization showed that HG20 RNA is highly expressed in the cortex, thalamus, hippocampus, and cerebellum of the brain, showing overlapping distribution with GABABR1a RNA as judged by *in situ* hybridization as well as with the expression of GABAB receptors as judged by pharmacological studies. HG20 RNA exhibits restricted distribution in the periphery, with low abundance of the 6.5 kb RNA in the heart, spleen, and pancreas and high levels in the adrenal gland. HG20 recombinantly expressed in COS-1 cells showed no specific binding for [<sup>3</sup>H](+)-baclofen, and when expressed in *Xenopus* oocyte and *Xenopus* melanophore functional assays, showed no activity to GABA,

(-)baclofen, and glutamic acid.

The novel DNA sequences of the present invention encoding HG20, in whole or in part, can be linked with other DNA sequences, *i.e.*, DNA sequences to which HG20 is not naturally linked, to form  
5 "recombinant DNA molecules" containing HG20. Such other sequences can include DNA sequences that control transcription or translation such as, *e.g.*, translation initiation sequences, promoters for RNA polymerase II, transcription or translation termination sequences, enhancer  
10 sequences, sequences that control replication in microorganisms, or that confer antibiotic resistance. The novel DNA sequences of the present invention can be inserted into vectors such as plasmids, cosmids, viral vectors, or yeast artificial chromosomes.

The present invention also includes isolated forms of DNA encoding HG20. By "isolated DNA encoding HG20" is meant DNA  
15 encoding HG20 that has been isolated from a natural source or produced by recombinant means. Use of the term "isolated" indicates that DNA encoding HG20 is not present in its normal cellular environment. Thus, an isolated DNA encoding HG20 may be in a cell-free solution or placed in  
20 a different cellular environment from that in which it occurs naturally. The term isolated does not imply that isolated DNA encoding HG20 is the only DNA present, but instead means that isolated DNA encoding HG20 is at least 95% free of non-nucleic acid material (*e.g.*, proteins, lipids,  
25 carbohydrates) naturally associated with the DNA encoding HG20. Thus, DNA encoding HG20 that is expressed in bacteria or even in eukaryotic cells which do not naturally (*i.e.*, without human intervention) contain it through recombinant means is "isolated DNA encoding HG20."

Included in the present invention are DNA sequences that hybridize to SEQ.ID.NO.:1 under stringent conditions. By way of  
30 example, and not limitation, a procedure using conditions of high stringency is as follows: Prehybridization of filters containing DNA is carried out for 2 hr. to overnight at 65°C in buffer composed of 6X SSC, 5X Denhardt's solution, and 100 µg/ml denatured salmon sperm DNA. Filters are hybridized for 12 to 48 hrs at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10<sup>6</sup> cpm

of  $^{32}\text{P}$ -labeled probe. Washing of filters is done at 37°C for 1 hr in a solution containing 2X SSC, 0.1% SDS. This is followed by a wash in 0.1X SSC, 0.1% SDS at 50°C for 45 min. before autoradiography.

Other procedures using conditions of high stringency would  
5 include either a hybridization carried out in 5XSSC, 5X Denhardt's solution, 50% formamide at 42°C for 12 to 48 hours or a washing step carried out in 0.2X SSPE, 0.2% SDS at 65°C for 30 to 60 minutes.

Reagents mentioned in the foregoing procedures for carrying  
out high stringency hybridization are well known in the art. Details of  
10 the composition of these reagents can be found in, e.g., Sambrook, Fritsch, and Maniatis, 1989, Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press. In addition to the foregoing, other conditions of high stringency which may be used are well known in the art.

15 Another aspect of the present invention includes host cells that have been engineered to contain and/or express DNA sequences encoding HG20. Such recombinant host cells can be cultured under suitable conditions to produce HG20. An expression vector containing DNA encoding HG20 can be used for expression of HG20 in a recombinant  
20 host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Cell lines  
25 derived from mammalian species which are suitable for recombinant expression of HG20 and which are commercially available, include but are not limited to, L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, and *Xenopus* oocytes. In particular  
30 embodiments, the recombinant cells expressing HG20 protein co-express a GABABR1a or GABABR1b protein, thus forming a functional GABAB



receptor comprising a heterodimer of HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b. In particular embodiments, the recombinant cells have been transfected with expression vectors that direct the expression of HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b.

5                   Cells that are particularly suitable for expression of the HG20 protein are melanophore pigment cells from *Xenopus laevis*. Such melanophore pigment cells can be used for functional assays that employ recombinant expression of HG20 in a manner similar to the use of such melanophore pigment cells for the functional assay of other recombinant  
10   GPCRs (Graminski et al., 1993, J. Biol. Chem. 268:5957-5964; Lerner, 1994, Trends Neurosci. 17:142-146; Potenza & Lerner, 1992, Pigment Cell Res. 5:372-378; Potenza et al., 1992, Anal. Biochem. 206:315-322). Especially preferred are *Xenopus* melanophore pigment cells co-expressing HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, in which HG20 has formed a  
15   heterodimer with GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, thus forming a functional GABA<sub>B</sub> receptor. The presence of functional GABA<sub>B</sub> receptors in such cells can be determined by the use of assays such as the pigment aggregation assay described herein. Other assays that reflect a decrease in cAMP levels mediated by exposure to GABA or other agonists of  
20   GABA<sub>B</sub> receptors would also be suitable.

                  Also preferred are stably or transiently transfected HEK293 cells co-expressing HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, in which HG20 has formed a heterodimer with GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, thus forming a functional GABA<sub>B</sub> receptor. The presence of functional GABA<sub>B</sub> receptors  
25   in such cells can be determined by the use of assays such as those that measure cAMP levels as described herein.

                  Also preferred are *Xenopus* oocytes co-expressing HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, in which HG20 has formed a heterodimer with GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, thus forming a functional GABA<sub>B</sub>  
30   receptor. The presence of functional GABA<sub>B</sub> receptors in such cells can be determined by the use of assays that measure coupling of functional GABA<sub>B</sub> receptors comprising heterodimers of HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b to inwardly rectifying potassium channels (especially the Kir3 family).

In order to produce the above-described cells co-expressing HG20 and GABABR1a or GABABR1b, expression vectors comprising DNA encoding HG20 and GABABR1a or GABABR1b can be transfected into the cells. HG20 and GABABR1a or GABABR1b can be transfected  
5 separately, each on its own expression vector, or, alternatively, a single expression vector encoding both HG20 and GABABR1a or GABABR1b can be used.

A variety of mammalian expression vectors can be used to express recombinant HG20, GABABR1a, or GABABR1b in mammalian  
10 cells. Commercially available mammalian expression vectors which are suitable include, but are not limited to, pMC1neo (Stratagene), pSG5 (Stratagene), pcDNAI and pcDNAIamp, pcDNA3, pcDNA3.1, pCR3.1 (Invitrogen), EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199),  
15 pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), and the PT7TS oocyte expression vector (or similar expression vectors containing the globin 5' UTR and the globin 3' UTR). The choice of vector will depend upon cell type used, level of expression desired, and the like. Following expression in recombinant cells, HG20, GABABR1a, GABABR1b, or heterodimers of  
20 HG20 and either GABABR1a or GABABR1b can be purified to a level that is substantially free from other proteins by conventional techniques, *e.g.*, salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography, hydrophobic interaction chromatography, and preparative gel  
25 electrophoresis. Also, membrane preparations comprising HG20, GABABR1a, GABABR1b, or heterodimers of HG20 and either GABABR1a or GABABR1b can be prepared. Especially preferred are membrane preparations that comprise heterodimers of HG20 and either GABABR1a or GABABR1b in which the heterodimers represent functional GABAB  
30 receptors.

The present invention includes a method of producing HG20 protein comprising:

(a) transfecting a host cell with an expression vector comprising DNA that encodes an HG20 protein;

(b) growing the host cells under conditions such that HG20 protein is produced; and

(c) recovering HG20 protein from the host cells.

In particular embodiments, the method of recovering HG20 protein involves obtaining membrane preparations that contain HG20 protein from the host cells. In particular embodiments, such membrane preparations contain heterodimers of HG20 protein and GABABR1a or GABABR1b protein that form functional GABAB receptors.

The present invention includes a method of expressing a truncated HG20 protein comprising:

(a) transfecting a host cell with an expression vector comprising DNA that encodes an HG20 protein that has been truncated at the amino or carboxyl terminus;

(b) culturing the transfected cells of step (a) under conditions such that the truncated HG20 protein is expressed.

Truncated HG20 proteins are those HG20 proteins in which contiguous portions of the N terminus or C terminus have been removed. For example, positions 52-941 of SEQ.ID.NO.:2 represents a truncated HG20 protein. Truncated HG20 proteins may be fused in frame to non-HG20 amino acid sequences, as, *e.g.*, in the FLAG-HG20 construct described herein.

The present invention includes a method of producing functional GABAB receptors in cells comprising:

(a) transfecting cells with:

(1) an expression vector that directs the expression of HG20 in the cells; and

(2) an expression vector that directs the expression of GABABR1a or GABABR1b in the cells;

(b) culturing the cells under conditions such that heterodimers of HG20 and GABABR1a or GABABR1b are formed where the heterodimers constitute functional GABAB receptors.

In particular embodiments of the above methods, the cells are eukaryotic cells. In other embodiments, the cells are mammalian cells. In still other embodiments, the cells are COS cells, *e.g.*, COS-7 cells

(ATCC CRL 1651) or COS-1 cells (ATCC CRL 1650); HEK293 cells (ATCC CRL 1573); or *Xenopus* melanophores.

In particular embodiments, the HG20 protein comprises the amino acid sequence shown in SEQ.ID.NO.:2. In particular embodiments, the HG20 protein is a truncated HG20 protein. In particular  
5       embodiments, the truncated HG20 protein comprises amino acids 52-941 of SEQ.ID.NO.:2. In particular embodiments, the truncated HG20 protein is a chimeric HG20 protein.

The present invention includes HG20 protein substantially  
10       free from other proteins. The amino acid sequence of the full-length HG20 protein is shown in Figure 2 as SEQ.ID.NO.:2. Thus, the present invention includes polypeptides comprising HG20 protein substantially free from other proteins where the polypeptides comprise the amino acid sequence SEQ.ID.NO.:2. The present invention also includes polypeptides  
15       comprising HG20 proteins lacking a signal sequence. Examples of amino acid sequences of HG20 proteins lacking a signal sequence are:

Positions 9-941 of SEQ.ID.NO.:2;  
Positions 35-941 of SEQ.ID.NO.:2;  
Positions 36-941 of SEQ.ID.NO.:2;  
20       Positions 38-941 of SEQ.ID.NO.:2;  
Positions 39-941 of SEQ.ID.NO.:2;  
Positions 42-941 of SEQ.ID.NO.:2;  
Positions 44-941 of SEQ.ID.NO.:2;  
Positions 46-941 of SEQ.ID.NO.:2;  
25       Positions 52-941 of SEQ.ID.NO.:2; and  
Positions 57-941 of SEQ.ID.NO.:2.

The present invention also includes DNA encoding the above-described HG20 proteins lacking a signal sequence. Thus, e.g., the present invention includes a DNA molecule comprising a nucleotide  
30       sequence selected from the group consisting of:

Positions 293-3,115 of SEQ.ID.NO.:1;  
Positions 317-3,115 of SEQ.ID.NO.:1;  
Positions 395-3,115 of SEQ.ID.NO.:1;  
Positions 398-3,115 of SEQ.ID.NO.:1;

Positions 404-3,115 of SEQ.ID.NO.:1;  
Positions 407-3,115 of SEQ.ID.NO.:1;  
Positions 416-3,115 of SEQ.ID.NO.:1;  
Positions 422-3,115 of SEQ.ID.NO.:1;  
5 Positions 428-3,115 of SEQ.ID.NO.:1;  
Positions 446-3,115 of SEQ.ID.NO.:1; and  
Positions 461-3,115 of SEQ.ID.NO.:1.

As with many receptor proteins, it is possible to modify many  
of the amino acids of HG20, particularly those which are not found in the  
10 ligand binding domain, and still retain substantially the same biological  
activity as the original protein. Thus this invention includes modified  
HG20 polypeptides which have amino acid deletions, additions, or  
substitutions but that still retain substantially the same biological  
activity as native HG20. It is generally accepted that single amino acid  
15 substitutions do not usually alter the biological activity of a protein (see,  
*e.g.*, Molecular Biology of the Gene, Watson et al., 1987, Fourth Ed., The  
Benjamin/Cummings Publishing Co., Inc., page 226; and Cunningham &  
Wells, 1989, Science 244:1081-1085). Accordingly, the present invention  
includes polypeptides where one amino acid substitution has been made  
20 in SEQ.ID.NO.:2 or in one of the HG20 polypeptides lacking a signal  
sequence listed above, wherein the polypeptides still retain substantially  
the same biological activity as native HG20. The present invention also  
includes polypeptides where two or more amino acid substitutions have  
been made in SEQ.ID.NO.:2 or in one of the HG20 polypeptides lacking a  
25 signal sequence listed above, wherein the polypeptides still retain  
substantially the same biological activity as native HG20. In particular,  
the present invention includes embodiments where the above-described  
substitutions are conservative substitutions. In particular, the present  
invention includes embodiments where the above-described substitutions  
30 do not occur in the ligand-binding domain of HG20. In particular, the  
present invention includes embodiments where amino acid changes have  
been made in the positions of HG20 where the amino acid sequence of  
HG20 differs from the amino acid sequence of GABABR1b (see Figure 8).

The present invention also includes C-terminal truncated forms of HG20, particularly those which encompass the extracellular portion of the receptor, but lack the intracellular signaling portion of the receptor. Such truncated receptors are useful in various binding assays described herein, for crystallization studies, and for structure-activity-relationship studies. Accordingly, the present invention includes an HG20 protein substantially free from other proteins having the amino acid sequence of positions 1-480 of SEQ.ID.NO.:2.

O'Hara et al., 1993, Neuron 11:41-52 (O'Hara) reported that the amino terminal domains of several metabotropic glutamate receptors showed amino acid sequence similarities to the amino termini of several bacterial periplasmic binding proteins. O'Hara used this similarity to predict, and then experimentally confirm, that these amino terminal domains correspond to the location of the ligand binding domains of these metabotropic glutamate receptors.

The present inventors have discovered a region of amino acid sequence in the N-terminal domain of HG20 that is similar to the amino acid sequence of the bacterial periplasmic binding protein Leucine, Isoleucine, Valine (Alanine and Threonine) Binding Protein (LIVAT-BP) of *Pseudomonas aeruginosa*. See Figure 6. The region shown is about 25% identical between the two proteins. This is above the maximum identity of 17% reported by O'Hara between any one metabotropic glutamate receptor and any one periplasmic binding protein and indicates that the region of HG20 depicted is highly likely to contain the ligand binding domain.

Accordingly, the present invention includes a polypeptide representing the ligand binding domain of HG20 that includes amino acids 63-259 of SEQ.ID.NO.:2. Also provided are chimeric proteins comprising amino acids 63-259 of SEQ.ID.NO.:2.

Romano et al., 1996, J. Biol. Chem. 271:28612-28616 demonstrated that metabotropic glutamate receptors are often found as homodimers formed by an intermolecular disulfide bond. The location of the cysteines responsible for the disulfide bond was found to be in the amino terminal 17kD of the receptors. Transmembrane interactions may

also contribute to functional GABA<sub>B</sub> receptor dimer formation, as previously reported for the dopamine D2 receptor and  $\beta$ 2-adrenergic receptor (Ng et al., 1996, Biochem. Biophys. Res. Comm. 227:200-204; Hebert et al., 1996, J. Biol. Chem. 271, 16384-16392). Accordingly, the present invention includes dimers of HG20 proteins. In particular  
5       embodiments, the HG20 protein has an amino acid selected from the group consisting of:

                    SEQ.ID.NO.:2;  
                    Positions 9-941 of SEQ.ID.NO.:2;  
10                   Positions 35-941 of SEQ.ID.NO.:2;  
                    Positions 36-941 of SEQ.ID.NO.:2;  
                    Positions 38-941 of SEQ.ID.NO.:2;  
                    Positions 39-941 of SEQ.ID.NO.:2;  
                    Positions 42-941 of SEQ.ID.NO.:2;  
15                   Positions 44-941 of SEQ.ID.NO.:2;  
                    Positions 46-941 of SEQ.ID.NO.:2;  
                    Positions 52-941 of SEQ.ID.NO.:2;  
                    Positions 57-941 of SEQ.ID.NO.:2; and  
                    Positions 1-480 of SEQ.ID.NO.:2.

20                   It has been found that, in some cases, membrane spanning regions of receptor proteins can be used to inhibit receptor function (Ng et al., 1996, Biochem. Biophys. Res. Comm. 227:200-204; Hebert et al., 1996, J. Biol. Chem. 271, 16384-16392; Lofts et al., Oncogene 8:2813-2820). Accordingly, the present invention provides peptides derived from the  
25       seven membrane spanning regions of HG20 and their use to inhibit HG20 or GABA<sub>B</sub> receptor function. Such peptides can include the whole or parts of the membrane spanning domains.

                    The present invention also includes isolated forms of HG20 proteins. By "isolated HG20 protein" is meant HG20 protein that has  
30       been isolated from a natural source or produced by recombinant means. Use of the term "isolated" indicates that HG20 protein is not present in its normal cellular environment. Thus, an isolated HG20 protein may be in a cell-free solution or placed in a different cellular environment from that in which it occurs naturally. The term isolated does not imply that an

isolated HG20 protein is the only protein present. but instead means that an isolated HG20 protein is at least 95% free of non-amino acid material (e.g., nucleic acids, lipids, carbohydrates) naturally associated with the HG20 protein. Thus, an HG20 protein that is expressed through  
5 recombinant means in bacteria or even in eukaryotic cells which do not naturally (i.e., without human intervention) express it is an "isolated HG20 protein."

The present invention also includes chimeric HG20 proteins. By chimeric HG20 protein is meant a contiguous polypeptide sequence of  
10 HG20 fused in frame to a polypeptide sequence of a non-HG20 protein. For example, the N-terminal domain and seven transmembrane spanning domains of HG20 fused at the C-terminus in frame to a G protein would be a chimeric HG20 protein. Another example of a chimeric HG20 protein would be a polypeptide comprising the FLAG epitope fused in frame at the  
15 amino terminus of amino acids 52-941 of SEQ.ID.NO.:2.

The present invention also includes HG20 proteins that are in the form of multimeric structures, e.g., dimers. Such multimers of other metabotropic G-protein coupled receptors are known (Hebert et al., 1996, J. Biol. Chem. 271, 16384-16392; Ng et al., 1996, Biochem. Biophys.  
20 Res. Comm. 227, 200-204; Romano et al., 1996, J. Biol. Chem. 271, 28612-28616).

Preferred forms of dimers of HG20 are heterodimers comprising HG20 and other G-protein coupled receptors (GPCRs). Such GPCRs could be, e.g., other subunits of GABA<sub>B</sub> receptors, proteins from *C. elegans* showing homology to HG20 (see Figure 24), or human GPCRs that  
25 are homologs of the *C. elegans* proteins. Particularly preferred forms of heterodimers are heterodimers of HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b. It has been found by the present inventors that such heterodimers exhibit functional properties of GABA<sub>B</sub> receptors while  
30 monomers or homodimers of HG20, GABA<sub>B</sub>R1a, or GABA<sub>B</sub>R1b do not exhibit functional properties. Another likely heterodimer partner for HG20 is the protein corresponding to the sequence deposited in GenBank at accession number 3776096.



The strongest evidence that functional GABA<sub>B</sub> receptors require both HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b comes from studies demonstrating that co-transfection and co-expression of both HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b is necessary in order for the detection of  
5 GABA<sub>B</sub> receptor functional responses. Transfection and expression of HG20, GABA<sub>B</sub>R1a, or GABA<sub>B</sub>R1b alone does not lead to the production of functional GABA<sub>B</sub> receptors.

For example, in *Xenopus* melanophores co-expressing HG20 and GABA<sub>B</sub>R1a, but not in melanophores expressing HG20 or  
10 GABA<sub>B</sub>R1a alone, or in mock transfected melanophores, GABA mediated a dose-dependent pigment aggregation response that could be inhibited with the GABA<sub>B</sub> receptor specific CGP71872 antagonist. This pigment aggregation response is associated with a decrease in intracellular cAMP levels. Such a decrease has been confirmed in HEK293 cells. Also, co-  
15 expression of HG20 and GABA<sub>B</sub>R1a in *Xenopus* oocytes resulted in the stimulation of inwardly rectifying potassium currents (Kirs). Native functional GABA<sub>B</sub> receptors have been reported to couple to Kirs (Misgeld et al., 1995, Prog. Neurobiol. 46:423-462).

Consistent with the need for both HG20 and GABA<sub>B</sub>R1a for  
20 detection of functional GABA<sub>B</sub> receptors in transfected cells, the present inventors have demonstrated that HG20 and GABA<sub>B</sub>R1a form heterodimers by immunoprecipitation of HG20 followed by immunoblotting with a GABA<sub>B</sub>R1a antibody.

That a functional GABA<sub>B</sub> receptor requires both HG20 and  
25 either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b is also suggested by the observation that GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, recombinantly expressed in the absence of HG20, binds ligand with much reduced affinity compared to the affinity of native GABA<sub>B</sub> receptors. Also, characterization of the tissue distribution of each of the receptors by *in situ* hybridization  
30 histochemistry in rat brain revealed co-localization of HG20 and GABA<sub>B</sub>R1a transcripts in many brain regions, including cortex, at both the regional and cellular levels.

The *Xenopus* melanophore pigment aggregation/dispersion assay has been shown to be highly suitable for monitoring agonist

activation of Gi-, Gq-, and Gs-coupled receptors (Potenza et al., 1992, Anal. Biochem. 206:315-322; Lerner, 1994, Trends Neurosci. 17:142-146). Agonist activation of Gi-coupled receptors expressed in melanophores results in pigment aggregation via a reduction in intracellular cAMP levels, whereas  
5 activation of Gs- and Gq-coupled receptors results in pigment dispersion via elevations in intracellular cAMP and calcium levels, respectively. Melanophores transfected separately with either GABABR1a or HG20 showed no pigment aggregation or dispersion response following treatment with up to 1 mM concentrations of (L)-glutamic acid, GABA, or  
10 prototypic GABAergic agonists: (-)baclofen, 3-aminopropyl-(methyl)phosphonic acid, cis-4-aminocrotonic acid, piperidine-4-sulfonic acid (data not shown). Similarly, both receptors failed to couple to K<sup>+</sup> channels in *Xenopus* oocytes under patch-clamp conditions when transfected separately (data not shown). However, in melanophores  
15 transiently co-transfected with GABABR1a and HG20, GABA mediated a dose-dependent aggregation response with an IC<sub>50</sub> value of 3-7  $\mu$ M (n=3). This aggregation was absent in mock-transfected cells and in cells transfected with GABABR1a or HG20 alone (Figure 11). The GABA-mediated activity represented 42-56% (n=3) of a control cannabinoid  
20 receptor subtype 2 response (Figure 11, inset), and could be inhibited by the CGP71872 antagonist (n=3), indicating it was GABAB receptor specific (Figure 11). GABABR1a was expressed by subcloning full-length GABABR1a into the NheI-NotI site of pcDNA3.1 or pCIneo; HG20 was expressed as a FLAG-HG20 chimeric protein. See Examples 11 and 20 for  
25 further experimental details of expression vectors used, transfection conditions, assay conditions, etc. for the above-described co-expression studies.

The functional data arising from co-expression of GABABR1a and HG20 receptors were confirmed in HEK293 cells. HEK293 cells  
30 transfected with and stably expressing GABABR1a and HG20 were selected based on expression of receptor message as determined by dot blot analyses. In cell lines stably expressing the individual receptors, we observed small and inconsistent responses in assays to examine agonist-mediated modulation of cAMP synthesis. However, transient transfection

of HEK293 cells stably expressing GABA<sub>B</sub>R1a (rgb1a-50) with an HG20 expression plasmid and transient transfection of HEK293 cells stably expressing HG20 (hgb2-42) with a GABA<sub>B</sub>R1a expression plasmid significantly enhanced the ability of baclofen and GABA to inhibit forskolin-stimulated cAMP synthesis. Rgb1a-50 cells transfected with HG20 exhibited a 28% reduction in forskolin-stimulated cAMP synthesis with 30  $\mu$ M baclofen and a 40% decrease with 30  $\mu$ M GABA plus 100  $\mu$ M aminooxyacetic acid (AOAA; a GABA transaminase inhibitor) and 100  $\mu$ M nipecotic acid (a GABA uptake inhibitor) (Figure 12B). A 34% reduction in forskolin-stimulated cAMP synthesis was observed for hgb2-42 cells transfected with GABA<sub>B</sub>R1a treated with baclofen and a 43% decrease was observed for GABA plus AOAA and nipecotic acid (Figure 12B). While inhibition of cAMP synthesis was sometimes observed with rgb1a-50 cells transfected with GABA<sub>B</sub>R1a and hgb2-42 cells transfected with HG20, these effects were small and inconsistent (0-20% inhibition; Figure 12B). Neither baclofen nor GABA plus AOAA and nipecotic acid in the absence of forskolin had any affect on cAMP synthesis (Figure 12B). In addition, wild-type HEK293 cells did not exhibit baclofen- or GABA-mediated inhibition of forskolin-stimulated cAMP synthesis (Figure 12B). These data demonstrate that the functional GABA<sub>B</sub> receptor requires both GABA<sub>B</sub>R1a and HG20. For experimental details of these studies in HEK293 cells, see Example 12.

Co-expression of the GABA<sub>B</sub>R1a and HG20 with the inwardly rectifying potassium channels Kir 3.1/3.2 in *Xenopus* oocytes resulted in a significant stimulation of inwardly rectifying potassium current (Kir) in response to GABA [301 +/- 20.6 %, (n=3) increase over control current] measured at -80 mV which could subsequently be washed out with control solution (Figure 13). Modulation of Kir 3.1/3.2 was not seen in oocytes expressing GABA<sub>B</sub>R1a or HG20 individually, even in the presence of Gi $\alpha$ 1 (Figure 13). See Example 21 for details.

To determine whether receptor intermolecular interactions accounted for the functional activity that was observed following the co-expression of recombinant GABA<sub>B</sub>R1a and HG20, membranes from cells co-expressing GABA<sub>B</sub>R1a and HG20 or the individual proteins were first

immunoprecipitated using anti-FLAG antibodies (to detect the recombinant FLAG-HG20 chimeric proteins) followed by immunoblotting with a GABA $\beta$ R1a-specific antibody. As seen in Figure 14, lanes 1-3, no GABA $\beta$ R1a immunoreactivity was detected in samples prepared from

5 mock vector transfected cells, FLAG-HG20 alone expressing cells, and GABA $\beta$ R1a alone expressing cells immunoprecipitated with the FLAG-antibody. Since immunoreactive species were detected only in cells co-expressing HG20 and GABA $\beta$ R1a, this experiment demonstrates that HG20 and GABA $\beta$ R1a can only be co-immunoprecipitated as part of a

10 complex (Figure 14, lane 4). Based on the predicted molecular mass of a heterodimer of HG20 and GABA $\beta$ R1a, the ~250+ and ~130 kDa species may represent a heterodimer and GABA $\beta$ R1a monomers, respectively. The stability of the HG20/GABA $\beta$ R1a heterodimer in denaturing and

15 reducing conditions suggests that SDS-stable transmembrane interactions form the heterodimer, as reported previously for  $\beta$ 2 adrenergic and dopamine D2 receptors (Ng et al., 1996, Biochem. Biophys. Res. Comm. 227:200-204; Hebert et al., 1996, J. Biol. Chem. 271, 16384-16392). The monomer might result from partial disruption, subsequent to

20 immunoprecipitation, of N-terminal Sushi repeats, C-terminal alpha-helical interacting domains (e.g., coiled-coils) present in HG20 and GABA $\beta$ R1a subunits, transmembrane interactions, or disulfide bonds that contribute to forming the heterodimer.

Particular examples of such regions likely to be involved in forming the heterodimer are shown in Figure 23. Regions such as those

25 shown in Figure 23, as well as polypeptides comprising such regions are expected to be useful for the purpose of modulating the formation of heterodimers involving HG20 and thus controlling GABA $\beta$  receptor activity. Accordingly, the present invention includes polypeptides comprising the coiled-coil domains of HG20, GABA $\beta$ R1a, and GABA $\beta$ R1b.

30 In particular, the present invention includes polypeptides comprising an amino acid sequence selected from the group consisting of: positions 756-829 of SEQ.ID.NO.:2; positions 779-814 of SEQ.ID.NO.:2; positions 886-949 of SEQ.ID.NO.:21; and positions 889-934 of SEQ.ID.NO.:21; where the polypeptides do not contain other contiguous amino acid sequences

longer than 5 amino acids from a GABA<sub>B</sub> receptor subunit. The present invention also includes heterodimers of such polypeptides. In more general terms, the present invention includes comprising a coiled-coil domain from a first GABA<sub>B</sub> receptor subunit and no other contiguous amino acid sequences longer than 5 amino acids from the first GABA<sub>B</sub> receptor subunit where the coiled-coil domain is present in the C-terminus of the first GABA<sub>B</sub> receptor subunit and mediates heterodimerization of the first GABA<sub>B</sub> receptor subunit with a second GABA<sub>B</sub> receptor subunit.

In addition to the coiled-coil domains discussed above, a variety of regions of HG20 and GABA<sub>B</sub>R1a are expected to be important for heterodimer formation. Motif analysis of the N-terminus of murine GABA<sub>B</sub>R1a revealed seven consensus N-linked glycosylation sites and three putative short consensus repeats (SCRs) of ~60 amino acids each: amino acids 27-96 and amino acids 102-157 (GABA<sub>B</sub>R1a specific), and amino acids 183-245 (common to GABA<sub>B</sub>R1b (Kaupmann et al., 1997; Nature 386:239-246) and HG20 (Jones et al., 1998, Nature 396:674-679; White et al., 1998, Nature 396:679-682; Kaupmann et al., 1998, Nature 396:683-687; Kuner et al., 1999, Science 283:74-77) not described previously (Figure 26A-B). Since SCRs are known to play important roles in protein-protein interactions in a wide variety of complement proteins, adhesion proteins, and selectins (Chou and Heinrikson, 1997, J. Protein Chem. 16:765-773; Perkins et al., 1998, Biochemistry 27:4004-4012), of which the latter shows weak amino acid identity to murine GABA<sub>B</sub>R1a, these SCRs, together with the coiled-coil domains discussed above in the carboxyl tails of GABA<sub>B</sub>R1a and HG20 (Figure 23), are expected to be involved in the heterodimerization of GABA<sub>B</sub>R1a and HG20.

Therefore, the present invention includes a polypeptide comprising an SCR domain from a first GABA<sub>B</sub> receptor subunit and no other contiguous amino acid sequences longer than 5 amino acids from the first GABA<sub>B</sub> receptor subunit where the SCR domain is present in the N-terminus of the first GABA<sub>B</sub> receptor subunit and mediates heterodimerization of the first GABA<sub>B</sub> receptor subunit with a second GABA<sub>B</sub> receptor subunit. In particular embodiments, the SCR is selected from the group consisting of: positions 27-96 of SEQ.ID.NO.:20; positions

102-157 of SEQ.ID.NO.:20; positions 183-245 of SEQ.ID.NO.:20; positions 28-97 of SEQ.ID.NO.:21; positions 103-158 of SEQ.ID.NO.:21; positions 184-246 of SEQ.ID.NO.:21; positions 4-22 of SEQ.ID.NO.:2; positions 23-49 of SEQ.ID.NO.:2; and positions 72-135 of SEQ.ID.NO.:2.

5           As in the metabotropic glutamate receptors (mGLURs), the second intracellular loop of murine GABABR1a is rich in basic amino acids which may play a role in G-protein-interactions (reviewed by Pin and Duvoisin, 1995, Neuropharmacology 34:1-26), and, as in the mGLURs, the carboxyl tail of murine GABABR1a contains a PDZ protein-  
10 interacting module (serine-arginine-valine, amino acids 953-955) which has been shown for mGLURs to play an important role in the interactions among the signaling components of synaptic junctions (Brakeman et al.1997, Nature 386:284-288). The murine GABABR1a receptor also contains potential protein kinase C and casein kinase II recognition sites  
15 predicted using ProSearch (Kolakowski et al., 1992, Biotechniques 13:919-921).

          The present invention also relates to the identification and cloning of the murine GABABR1a receptor, the murine ortholog of the rat GABABR1a receptor described in Kaupmann et al., 1997, Nature 386:239-  
20 246 (Kaupmann). The present invention provides DNA encoding murine GABABR1a that is substantially free from other nucleic acids. The present invention also provides recombinant DNA molecules encoding murine GABABR1a.

          The present invention provides a DNA molecule encoding  
25 murine GABABR1a that is substantially free from other nucleic acids and comprises the nucleotide sequence shown in Figure 15 as SEQ.ID.NO.:19. The open reading frame of SEQ.ID.NO.:19, encoding mouse GABABR1a protein, is positions 1-2,880, with positions 2,881-2,883 representing a stop codon. Thus, the present invention also provides a DNA molecule  
30 substantially free from other nucleic acids comprising the nucleotide sequence of positions 1-2,880 of SEQ.ID.NO.:19.

          Sequence analysis of the open reading frame of the murine GABABR1a DNA revealed that it encodes a mature protein (*i.e.*, lacking a signal sequence) of 942 amino acids with a predicted molecular weight of

encoding murine GABA<sub>B</sub>R1a. Such recombinant host cells can be cultured under suitable conditions to produce murine GABA<sub>B</sub>R1a protein. An expression vector containing DNA encoding the murine GABA<sub>B</sub>R1a protein can be used for expression of the murine GABA<sub>B</sub>R1a protein in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Cell lines derived from mammalian species which are suitable for recombinant expression of the murine GABA<sub>B</sub>R1a protein and which are commercially available, include but are not limited to, L cells L-M(TK-) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, and *Xenopus* oocytes.

A variety of mammalian expression vectors can be used to express recombinant murine GABA<sub>B</sub>R1a in mammalian cells. Commercially available mammalian expression vectors which are suitable include, but are not limited to, pMC1neo (Stratagene), pSG5 (Stratagene), pcDNA1 and pcDNA1amp, pcDNA3, pcDNA3.1, pCR3.1 (Invitrogen), EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pDBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), and the PT7TS oocyte expression vector (or similar expression vectors containing the globin 5' UTR and the globin 3' UTR). Following expression in recombinant cells, the murine GABA<sub>B</sub>R1a protein can be purified by conventional techniques to a level that is substantially free from other proteins.

Other cells that are particularly suitable for expression of the murine GABA<sub>B</sub>R1a protein are immortalized melanophore pigment cells from *Xenopus laevis*. Such melanophore pigment cells can be used for functional assays using recombinant expression of murine GABA<sub>B</sub>R1a in

106.5 kDa that is 99% identical to rat GABA<sub>B</sub>R1a (described in Kaupmann), with only six amino acid changes overall. Murine GABA<sub>B</sub>R1a protein shares 31% overall amino acid identity to HG20.

CGP71872 is a photoaffinity ligand specific for GABA<sub>B</sub>R1a receptors ( $K_d = 1.0 \pm 0.2$  nM) that can be cross-linked to rat GABA<sub>B</sub>R1a (Kaupmann et al., 1997, Nature 386:239-246). In crude membranes prepared from COS-7 cells transiently transfected with murine GABA<sub>B</sub>R1a, [<sup>125</sup>I]CGP71872 photolabelled a major band at ~130 kDa representing the mature (presumably glycosylated) protein and an additional band at approximately twice that molecular weight, possibly representing dimers (Figure 9). Ligand-binding species could also be detected with affinity purified GABA<sub>B</sub>R1a antibodies 1713.1 (raised against the peptide acetyl-DVNSRRDILPDYELKLC-amide; a portion of SEQ.ID.NO.:20) and 1713.2 (raised against the peptide acetyl-CATLHNPTRVKLF EK-amide; a portion of SEQ.ID.NO.:20) (Figure 9). In contrast, FLAG-tagged HG20 protein did not bind the high-affinity CGP71872 ligand, although expression of the protein was confirmed by immunoblot analysis (Figure 9).

Displacement of [<sup>125</sup>I]CGP71872 binding to recombinant murine GABA<sub>B</sub>R1a was in the appropriate rank order of potency for GABAergic ligands: CGP71872 > SKF-97541 (3-aminopropyl(methyl)-phosphinic acid) > GABA > (-)baclofen > saclofen > (L)-glutamic acid.. Interestingly, recombinant rat GABA<sub>B</sub>R1a exhibits 10-25 fold lower affinity for agonists than native GABA<sub>B</sub> receptors in brain (Kaupmann et al., 1997, Nature 386:239). Although the reason for this discrepancy remains unclear, a recent report indicated that recombinant GABA<sub>B</sub>R1a may require additional cellular components for functional targeting to the plasma membrane (Couve et al., 1998, J. Biol. Chem. 273:26361-26367). Thus, GABA<sub>B</sub>R1a alone, without such additional components, might be expected to exhibit somewhat altered ligand binding characteristics.

In the binding experiments discussed above using GABA<sub>B</sub>R1a alone, surprisingly, dose-dependent displacement was not detected for (+)baclofen, and the affinities of agonists (GABA, SKF-97541, and (-)baclofen) and partial agonists ((+)baclofen, saclofen, (L)-glutamic



acid) but not the affinity of antagonist (CGP71872) for the recombinant GABA<sub>B</sub>R1a were markedly lower compared to native receptors in rat brain (Table 1). Agonist affinities of co-expressed HG20 and GABA<sub>B</sub>R1a were examined in membranes prepared from cells co-expressing GABA<sub>B</sub>R1a and FLAG-tagged HG20. Competition of [<sup>125</sup>I]CGP71872 binding in these membranes showed recovery of high-affinity ligand binding comparable to native receptors in rat brain (Table 1). The simplest explanation for these results is that the high-affinity agonist binding pocket may comprise interactions between the N-terminal domains of HG20 and GABA<sub>B</sub>R1a that form the heterodimer.

Table 1

Ligand	rat cortex*	gb1a	gb1a/gb2
CGP71872	0.5 nM	0.52 - 0.67 nM	0.15 - 0.27 nM
GABA	2.5 $\mu$ M	42.55 - 68.38 $\mu$ M	1.77 - 2.55 $\mu$ M
SKF-97541**	not determined	11.09 - 11.47 $\mu$ M	0.80 - 0.96 $\mu$ M
(-)Baclofen	0.5 $\mu$ M	31.46 - 53.70 $\mu$ M	3.92 - 7.78 $\mu$ M
(+)Baclofen	not determined	no fit	1.25 - 3.94 mM
Saclofen	156 $\mu$ M	280.5 - 365.0 $\mu$ M	119.4 - 131.4 $\mu$ M
L-Glutamate	not determined	119.4 - 285.0 mM	116.2 - 201.6 mM

\* reported by Kaupmann et al., (1997) *Nature* 386, 239-246  
 \*\* 3-aminopropyl(methyl)phosphinic acid

In Table 1, gb1a refers to GABA<sub>B</sub>R1a and gb1a/gb2 refers to HG20/ GABA<sub>B</sub>R1a heterodimers.

Co-localization studies were performed to determine if mRNAs for GABA<sub>B</sub>R1a and HG20 co-exist in the same cells in the brain. Figure 10A-B shows equivalent levels of GABA<sub>B</sub>R1a and HG20 hybridization in adjacent coronal sections of rat parietal cortex, indicating that messages for both receptors are expressed in this brain region. Radiolabelled and fluorescent probes for the two receptors were used to look at the cellular level where it was observed that message for both receptors is expressed in the same cells (Example 13 and Figure 10C-E). In the parietal cortex and all other major brain regions studied, including the hippocampus, thalamus, cerebellum, and vestibular ganglion, GABA<sub>B</sub>R1a and HG20 mRNAs are co-localized in the same cells. These results suggest that the functional native GABA<sub>B</sub> receptors found in these

cells involve both GABA<sub>B</sub>R1a and HG20. Co-immunoprecipitation, functional, and anatomical data described herein converge to strongly support the argument that the native, functional GABA<sub>B</sub> receptor is a heterodimer of GABA<sub>B</sub>R1a and HG20. This work is particularly exciting  
5 because it represents the first example of a heteromeric G protein-coupled receptor.

The novel murine GABA<sub>B</sub>R1a DNA sequences of the present, in whole or in part, can be linked with other DNA sequences, *i.e.*, DNA sequences to which GABA<sub>B</sub>R1a DNA is not naturally linked, to form  
10 "recombinant DNA molecules" encoding murine GABA<sub>B</sub>R1a. Such other sequences can include DNA sequences that control transcription or translation such as, *e.g.*, translation initiation sequences, promoters for RNA polymerase II, transcription or translation termination sequences, enhancer sequences, sequences that control replication in microorganisms,  
15 or that confer antibiotic resistance. The novel DNA sequences of the present invention can be inserted into vectors such as plasmids, cosmids, viral vectors, or yeast artificial chromosomes.

The present invention also includes isolated forms of DNA encoding GABA<sub>B</sub>R1a. By "isolated DNA encoding GABA<sub>B</sub>R1a" is meant  
20 DNA encoding GABA<sub>B</sub>R1a that has been isolated from a natural source or produced by recombinant means. Use of the term "isolated" indicates that DNA encoding GABA<sub>B</sub>R1a is not present in its normal cellular environment. Thus, an isolated DNA encoding GABA<sub>B</sub>R1a may be in a cell-free solution or placed in a different cellular environment from that in  
25 which it occurs naturally. The term isolated does not imply that isolated DNA encoding GABA<sub>B</sub>R1a is the only DNA present, but instead means that isolated DNA encoding GABA<sub>B</sub>R1a is at least 95% free of non-nucleic acid material (*e.g.*, proteins, lipids, carbohydrates) naturally associated with the DNA encoding GABA<sub>B</sub>R1a. Thus, DNA encoding GABA<sub>B</sub>R1a  
30 that is expressed in bacteria or even in eukaryotic cells which do not naturally (*i.e.*, without human intervention) contain it through recombinant means is "isolated DNA encoding GABA<sub>B</sub>R1a."

Another aspect of the present invention includes host cells that have been engineered to contain and/or express DNA sequences

a manner similar to the use of such melanophore pigment cells for the functional assay of other recombinant GPCRs (Graminski et al., 1993, J. Biol. Chem. 268:5957-5964; Lerner, 1994, Trends Neurosci. 17:142-146; Potenza & Lerner, 1992, Pigment Cell Res. 5:372-378; Potenza et al.,  
5 1992, Anal. Biochem. 206:315-322).

The present invention includes a method of producing the murine GABA<sub>B</sub>R1a protein comprising:

- (a) transfecting a host cell with a expression vector comprising DNA that encodes the murine GABA<sub>B</sub>R1a protein;
- 10 (b) growing the host cells under conditions such that the murine GABA<sub>B</sub>R1a protein is produced; and
- (c) recovering the murine GABA<sub>B</sub>R1a protein from the host cells.

In particular embodiments, the method of recovering the  
15 murine GABA<sub>B</sub>R1a protein may involve obtaining membrane preparations from the host cells that contain the murine GABA<sub>B</sub>R1a protein. Such membrane preparations may contain heterodimers of GABA<sub>B</sub>R1a protein and HG20 protein that form functional GABA<sub>B</sub> receptors.

20 In particular embodiments, the cells are eukaryotic cells. In other embodiments, the cells are mammalian cells. In still other embodiments, the cells are COS cells, in particular COS-7 cells (ATCC CRL 1651), COS-1 cells (ATCC CRL 1650), HEK293 cells (ATCC CRL 1573), or *Xenopus* melanophores.

25 The present inventors have discovered that, when either HG20 or GABA<sub>B</sub>R1a subunits are recombinantly expressed separately, *i.e.*, in different cells, very little or no expression is observed. It is only when HG20 and GABA<sub>B</sub>R1a subunits are recombinantly co-expressed, *i.e.*, expressed in the same cells at the same time, that high level  
30 expression of HG20 and GABA<sub>B</sub>R1a is observed (see Figure 25). Given the close relationship among GABA<sub>B</sub>R1a, GABA<sub>B</sub>R1b, *C. elegans* genes related to GABA<sub>B</sub>R1a and HG20 (see Figure 24), and the close relationship that is expected to be found between other isoforms of GABA<sub>B</sub>R1a and GABA<sub>B</sub>R1b, it is believed that co-expression of HG20

and either GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b will also result in increased expression of HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b as compared to expression of these proteins separately.

Accordingly, the present invention includes a method of co-expressing HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b so as to result in an increase in expression of HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b as compared to expression when HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b are expressed separately. In particular embodiments, the level of expression of HG20, GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b is measured in the co-expressing cells. In particular embodiments, the level of expression of HG20, GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b is measured by immunoblot or by immunoprecipitation/immunoblotting methods.

Thus, the present invention includes a method of increasing expression of HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b comprising:

- (a) recombinantly expressing HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b in the same cells;
- (b) measuring the expression of HG20, GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b, where a measurement of detectable expression of HG20, GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes

related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b indicates that increased expression has been achieved.

In particular embodiments, the measurement of expression is carried out by immunoblotting with or without immunoprecipitation.

- 5 In other embodiments, the method also comprises the steps of recombinantly expressing HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b separately, measuring the level of expression of HG20, GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to
- 10 GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b in the separately expressing cells, and comparing the amount of expression of HG20, GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b in the separately expressing cells to the amount of expression of HG20,
- 15 GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b in the co-expressing cells.

- Accordingly, the present invention includes a a method of increasing expression of HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a
- 20 and GABA $\beta$ R1b comprising:

- (a) recombinantly expressing HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b in the same cells to form co-
- 25 expressing cells;
- (b) recombinantly expressing HG20 and GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b in different cells to form separately expressing cells;
- 30 (c) measuring the expression of HG20, GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other isoforms of GABA $\beta$ R1a and GABA $\beta$ R1b in the co-expressing cells;
- (d) measuring the expression of HG20, GABA $\beta$ R1a, GABA $\beta$ R1b, *C. elegans* genes related to GABA $\beta$ R1a and HG20, or other

isoforms of GABABR1a and GABABR1b in the separately expressing cells;

where if the amount of expression of HG20, GABABR1a, GABABR1b, *C. elegans* genes related to GABABR1a and HG20, or other isoforms of GABABR1a and GABABR1b is greater in the co-expressing cells as compared to the separately expressing cells, this indicates that increased expression has been achieved.

In particular embodiments, the measurement of expression is carried out by immunoblotting with or without immunoprecipitation.

10 The present invention includes murine GABABR1a protein substantially free from other proteins. The amino acid sequence of the full-length murine GABABR1a protein is shown in Figure 16 as SEQ.ID.NO.:20. Thus, the present invention includes polypeptides comprising the murine GABABR1a protein substantially free from other  
15 proteins having the amino acid sequence SEQ.ID.NO.:20. The present invention also includes murine GABABR1a protein lacking a signal sequence as well as DNA encoding such a protein. Such a murine GABABR1a protein lacking a signal sequence is represented by amino acids 18-960 of SEQ.ID.NO.:20.

20 The present invention includes modified murine GABABR1a polypeptides which have amino acid deletions, additions, or substitutions but that still retain substantially the same biological activity as native murine GABABR1a protein. The present invention includes polypeptides where one amino acid substitution has been made in SEQ.ID.NO.:20 or in  
25 a polypeptide represented by SEQ.ID.NO.:20 lacking a signal sequence, wherein the polypeptides still retain substantially the same biological activity as native murine GABABR1a protein. The present invention also includes polypeptides where two or more amino acid substitutions have been made in SEQ.ID.NO.:20 or in a polypeptide represented by  
30 SEQ.ID.NO.:20 lacking a signal sequence, wherein the polypeptides still retain substantially the same biological activity as native murine GABABR1a protein. In particular, the present invention includes embodiments where the above-described substitutions are conservative substitutions. In particular, the present invention includes embodiments

where the above-described substitutions do not occur in the ligand-binding domain of native murine GABABR1a protein. In particular, the present invention includes embodiments where amino acid changes have been made in positions of native murine GABABR1a protein where the amino acid sequence of native murine GABABR1a protein differs from the amino acid sequence of HG20 when the amino acid sequences of native murine GABABR1a protein and HG20 are aligned in a manner similar to the alignment of the amino acid sequences of GABABR1b protein and HG20 shown in Figure 8.

The present invention also includes isolated forms of murine GABABR1a proteins. By "isolated murine GABABR1a protein" is meant murine GABABR1a protein that has been isolated from a natural source or produced by recombinant means. Use of the term "isolated" indicates that murine GABABR1a protein is not present in its normal cellular environment. Thus, an isolated murine GABABR1a protein may be in a cell-free solution or placed in a different cellular environment from that in which it occurs naturally. The term isolated does not imply that an isolated murine GABABR1a protein is the only protein present, but instead means that an isolated murine GABABR1a protein is at least 95% free of non-amino acid material (e.g., nucleic acids, lipids, carbohydrates) naturally associated with the murine GABABR1a protein. Thus, an murine GABABR1a protein that is expressed in bacteria or even in eukaryotic cells which do not naturally (i.e., without human intervention) express it through recombinant means is an "isolated murine GABABR1a protein."

The present invention also provides ligand-binding domains of murine GABABR1a protein. A FASTA search of the database GenBank (bacterial division) using the N-terminal domain of murine GABABR1a (amino acid positions 147-551 of SEQ.ID.NO.:20) as the probe reveals a match with the *E.coli* leucine-specific binding protein (livK) (22% identity over 339 amino acids), whereas no match to any bacterial amino acid binding protein is found using the receptor sequence inclusive of the region that includes the seven transmembrane domains (TM 1-7; amino acid positions 552-960) as a probe. The ligand-binding domain(s) of

GABA<sub>B</sub>R1a was also experimentally determined. Photoaffinity [125I]CGP71872 labeling experiments provided direct physical evidence that the N-terminal extracellular domain but not a C-terminal fragment of GABA<sub>B</sub>R1a (comprising TM1-7 and inclusive to the carboxyl tail) is

5 responsible for ligand-binding (see Examples 14-19 and Figure 17B).

Accordingly, the present invention includes a polypeptide comprising the ligand binding domain of murine GABA<sub>B</sub>R1a. In preferred embodiments, the polypeptide comprises amino acids 147-551 of SEQ.ID.NO.:20.

10 The present invention includes methods of identifying compounds that specifically bind to the GABA<sub>B</sub> receptor, as well as compounds identified by such methods. The specificity of binding of compounds showing affinity for the GABA<sub>B</sub> receptor is shown by measuring the affinity of the compounds for recombinant cells expressing  
15 HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, or for membranes from such cells. Expression of the GABA<sub>B</sub> receptor and screening for compounds that bind to the GABA<sub>B</sub> receptor or that inhibit the binding of a known, radiolabeled ligand of the GABA<sub>B</sub> receptor, *e.g.*, an amino acid or a GABA analogue such as (-)baclofen, to these cells, or membranes prepared from  
20 these cells, provides an effective method for the rapid selection of compounds with high affinity for the GABA<sub>B</sub> receptor. Other radiolabeled ligands that might be used are ibotenic acid, the amino acids glutamate and glycine, other amino acids, decarboxylated amino acids, or any of the other GABA<sub>B</sub> receptor ligands disclosed herein or known in the art. Such  
25 ligands need not necessarily be radiolabeled but can also be nonisotopic compounds that can be used to displace bound radiolabeled compounds or that can be used as activators in functional assays. Compounds identified by the methods disclosed herein are likely to be agonists or antagonists of the GABA<sub>B</sub> receptor and may be peptides, proteins, or non-proteinaceous  
30 organic molecules.

Therefore, the present invention includes assays by which GABA<sub>B</sub> receptor agonists and antagonists can be identified. Methods for identifying agonists and antagonists of other receptors are well known in the art and can often be adapted to identify agonists and antagonists of



the GABA<sub>B</sub> receptor. Accordingly, the present invention includes a method for determining whether a substance binds GABA<sub>B</sub> receptors and is thus a potential agonist or antagonist of the GABA<sub>B</sub> receptor that comprises:

- 5 (a) providing cells comprising an expression vector encoding HG20 and an expression vector encoding GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b;
  - (b) culturing the cells under conditions such that HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b are expressed and heterodimers of HG20  
10 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b are formed;
  - (c) exposing the cells to a labeled ligand of GABA<sub>B</sub> receptors in the presence and in the absence of the substance;
  - (d) measuring the binding of the labeled ligand to the heterodimers of HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the presence and  
15 in the absence of the substance;
- where if the amount of binding of the labeled ligand is less in the presence of the substance than in the absence of the substance, then the substance is a potential agonist or antagonist of GABA<sub>B</sub> receptors.
- Examples of ligands of GABA<sub>B</sub> receptors are: CGP71872,  
20 GABA, saclofen, (-)baclofen, glycine, and (L)-glutamic acid.
- The present invention also includes a method for determining whether a substance is capable of binding to GABA<sub>B</sub> receptors, *i.e.*, whether the substance is a potential agonist or an antagonist of GABA<sub>B</sub> receptors, where the method comprises:
- 25 (a) providing test cells comprising an expression vector encoding HG20 and an expression vector encoding GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b;
  - (b) culturing the test cells under conditions such that HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b are expressed and heterodimers of  
30 HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b are formed;
  - (c) exposing the test cells to the substance;
  - (d) measuring the amount of binding of the substance to the test cells;

(e) measuring the amount of binding of the substance to control cells;

(f) comparing the amount of binding of the substance to the test cells with the amount of binding of the substance to control cells;

5 where if the amount of binding of the substance to the test cells is greater than the amount of binding of the substance to control cells, then the substance is capable of binding to GABA<sub>B</sub> receptors;

10 where the control cells are essentially the same as the test cells except that the control cells do not comprise an expression vector encoding HG20 and an expression vector encoding GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b.

15 Once a substance has been identified by the above-described methods, determining whether the substance is an agonist or antagonist can then be accomplished by the use of functional assays such as those described herein.

In particular embodiments, the cells are transfected with an expression vector encoding HG20 and an expression vector encoding GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b.

20 In particular embodiments, the binding affinity of the substance for the test cells is determined. In particular embodiments, such binding affinity is between 1nM and 200 mM; preferably between 5 nM and 1 mM; more preferably between 10 nM and 100 μM; and even more preferably between 10 nM and 100 nM.

25 The conditions under which step (c) of the above-described methods is practiced are conditions that are typically used in the art for the study of protein-ligand interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

30 In a particular embodiment of the above-described methods, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK-) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC

CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), or *Xenopus* melanophores.

5 The assays described above can be carried out with cells that have been transiently or stably transfected with an expression vector encoding HG20 and an expression vector encoding GABABR1a or GABABR1b. Transfection is meant to include any method known in the art for introducing HG20 and GABABR1a or GABABR1b into the test cells. For example, transfection includes calcium phosphate or calcium  
10 chloride mediated transfection, lipofection, infection with a retroviral construct, and electroporation. In particular embodiments, a single expression vector encodes HG20 and GABABR1a or GABABR1b.

Where binding of the substance or ligand is measured, such binding can be measured by employing a labeled substance or ligand. The  
15 substance or ligand can be labeled in any convenient manner known to the art, *e.g.*, radioactively, fluorescently, enzymatically.

In particular embodiments of the above-described methods, the substance or ligand is an amino acid or an amino acid analogue such as CGP71872, GABA, saclofen, (-)-baclofen, glycine, and (L)-glutamic acid.

20 In particular embodiments of the above-described methods, HG20 has an amino acid sequence of SEQ.ID.NO.:2.

In particular embodiments of the above-described methods, HG20 comprises an amino acid sequence selected from the group consisting of:

25 SEQ.ID.NO.:2;  
Positions 9-941 of SEQ.ID.NO.:2;  
Positions 35-941 of SEQ.ID.NO.:2;  
Positions 36-941 of SEQ.ID.NO.:2;  
Positions 38-941 of SEQ.ID.NO.:2;  
30 Positions 39-941 of SEQ.ID.NO.:2;  
Positions 42-941 of SEQ.ID.NO.:2;  
Positions 44-941 of SEQ.ID.NO.:2;  
Positions 46-941 of SEQ.ID.NO.:2;  
Positions 52-941 of SEQ.ID.NO.:2; and

Positions 57-941 of SEQ.ID.NO.:2.

In particular embodiments, GABA<sub>B</sub>R1a is murine GABA<sub>B</sub>R1a and has the amino acid sequence SEQ.ID.NO.:20. In particular embodiments, GABA<sub>B</sub>R1a is rat GABA<sub>B</sub>R1a and has the  
5 amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In particular embodiments, GABA<sub>B</sub>R1b is rat GABA<sub>B</sub>R1b and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In particular embodiments, GABA<sub>B</sub>R1a is human GABA<sub>B</sub>R1a and has an amino acid sequence selected from the group  
10 consisting of: SEQ.ID.NO.:21 and the protein encoded by SEQ.ID.NO.:23.

The above-described methods can be modified in that, rather than exposing cells to the substance, membranes can be prepared from the cells and those membranes can be exposed to the substance. Such a modification utilizing membranes rather than cells is well known in the  
15 art with respect to other receptors and is described in, *e.g.*, Hess et al., 1992, Biochem. Biophys. Res. Comm. 184:260-268.

As a further modification of the above-described method, RNA encoding HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b can be prepared as, *e.g.*, by *in vitro* transcription using a plasmid containing HG20 and a  
20 plasmid containing GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b under the control of a bacteriophage T7 promoter, and the RNA can be microinjected into *Xenopus* oocytes in order to cause the expression of HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the oocytes. Substances are then tested for binding to the heterodimer of HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b expressed in the  
25 oocytes. Alternatively, rather than detecting binding, the effect of the substances on the electrophysiological properties of the oocytes can be determined.

The present invention includes assays by which GABA<sub>B</sub> receptor agonists and antagonists may be identified by their ability to  
30 stimulate or antagonize a functional response mediated by the GABA<sub>B</sub> receptor in cells that have been co-transfected with and that co-express HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b.

Accordingly, the present invention provides a method of identifying agonists and antagonists of HG20 comprising:

- (a) providing test cells by transfecting cells with:
- (1) an expression vector that directs the expression of HG20 in the cells; and
- (2) an expression vector that directs the expression of GABABR1a or GABABR1b in the cells;
- (b) exposing the test cells to a substance that is suspected of being an agonist of the GABAB receptor;
- (c) measuring the amount of a functional response of the test cells that have been exposed to the substance;
- (d) comparing the amount of the functional response exhibited by the test cells with the amount of the functional response exhibited by control cells;
- wherein if the amount of the functional response exhibited by the test cells differs from the amount of the functional response exhibited by the control cells, the substance is an agonist or antagonist of the GABAB receptor;
- where the control cells are cells that have not been transfected with HG20 and GABABR1a or GABABR1b but have been exposed to the substance or are test cells that have not been exposed to the substance.
- In particular embodiments of the above-described methods, HG20 has an amino acid sequence of SEQ.ID.NO.:2.
- In particular embodiments of the above-described methods, HG20 comprises an amino acid sequence selected from the group consisting of:
- SEQ.ID.NO.:2;
- Positions 9-941 of SEQ.ID.NO.:2;
- Positions 35-941 of SEQ.ID.NO.:2;
- Positions 36-941 of SEQ.ID.NO.:2;
- Positions 38-941 of SEQ.ID.NO.:2;
- Positions 39-941 of SEQ.ID.NO.:2;
- Positions 42-941 of SEQ.ID.NO.:2;
- Positions 44-941 of SEQ.ID.NO.:2;
- Positions 46-941 of SEQ.ID.NO.:2;

Positions 52-941 of SEQ.ID.NO.:2; and

Positions 57-941 of SEQ.ID.NO.:2.

In particular embodiments, GABA<sub>B</sub>R1a is murine GABA<sub>B</sub>R1a and has the amino acid sequence SEQ.ID.NO.:20. In  
5 particular embodiments, GABA<sub>B</sub>R1a is rat GABA<sub>B</sub>R1a and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In particular embodiments, GABA<sub>B</sub>R1b is rat GABA<sub>B</sub>R1b and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In particular embodiments, GABA<sub>B</sub>R1a is human  
10 GABA<sub>B</sub>R1a and has an amino acid sequence selected from the group consisting of: SEQ.ID.NO.:21 and the protein encoded by SEQ.ID.NO.:23.

In particular embodiments, the functional response is selected from the group consisting of: changes in pigment distribution in melanophore cells; changes in cAMP or calcium concentration; and  
15 changes in membrane currents in *Xenopus* oocytes. In particular embodiments, the change in pigment distribution is pigment aggregation; the change in cAMP concentration is a decrease in cAMP concentration; the change in membrane current is the modulation of an inwardly rectifying potassium current.

20 In a particular embodiment of the above-described method, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK-) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650),  
25 COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, or *Xenopus* oocytes.

In a particular embodiment of the above-described method,  
30 the cells are transfected with separate expression vectors that direct the expression of HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the cells. In other embodiments, the cells are transfected with a single expression vector that direct the expression of both HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the cells.

In a particular embodiment, the cells are *Xenopus* melanophores and the functional response is pigment aggregation. In another embodiment, the cells are HEK293 cells and the functional response is a decrease in cAMP level. In another embodiment, the cells are *Xenopus* oocytes and the functional response is the production of an inwardly rectifying potassium current.

The conditions under which step (b) of the method is practiced are conditions that are typically used in the art for the study of protein-ligand interactions: e.g., physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

The above-described assay can be easily modified to form a method to identify antagonists of the GABA<sub>B</sub> receptor. Such a method comprises:

- 15 (a) providing cells by transfecting cells with:
  - (1) an expression vector that directs the expression of HG20 in the cells; and
  - (2) an expression vector that directs the expression of GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the cells;
- 20 (b) exposing the cells to a substance that is a known agonist of the GABA<sub>B</sub> receptor;
- (c) measuring the amount of a functional response of the cells that have been exposed to the known agonist;
- (d) exposing the cells concurrently to the known agonist and to a substance that is suspected of being an antagonist of the GABA<sub>B</sub> receptor;
- 25 (e) measuring the amount of a functional response of the cells that have been exposed to the substance and the known agonist;
- (f) comparing the amount of the functional response measured in step (c) with the amount of the functional response measured in step (e);
- 30

wherein if the amount of the functional response measured in step (c) is greater than the amount of the functional response measured in step (e), the substance is an antagonist of the GABA<sub>B</sub> receptor.

Additional types of functional assays that can be used to identify agonists and antagonists of GABA<sub>B</sub> receptors include transcription-based assays. Transcription-based assays involve the use of a reporter gene whose transcription is driven by an inducible promoter whose activity is regulated by a particular intracellular event such as, *e.g.*, changes in intracellular calcium levels that are caused by the interaction of a receptor with a ligand. Transcription-based assays are reviewed in Rutter et al., 1998, *Chemistry & Biology* 5:R285-R290.

The transcription-based assays of the present invention rely on the expression of reporter genes whose transcription is activated or repressed as a result of intracellular events that are caused by the interaction of an agonist with a heterodimer of HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b where the heterodimer forms a functional GABA<sub>B</sub> receptor.

An extremely sensitive transcription based assay is disclosed in Zlokarnik et al., 1998, *Science* 279:84-88 (Zlokarnik) and also in U.S. Patent No. 5,741,657. The assay disclosed in Zlokarnik and U.S. Patent No. 5,741,657 employs a plasmid encoding  $\beta$ -lactamase under the control of an inducible promoter. This plasmid is transfected into cells together with a plasmid encoding a receptor for which it is desired to identify agonists. The inducible promoter on the  $\beta$ -lactamase is chosen so that it responds to at least one intracellular signal that is generated when an agonist binds to the receptor. Thus, following such binding of agonist to receptor, the level of  $\beta$ -lactamase in the transfected cells increases. This increase in  $\beta$ -lactamase is made measurable by treating the cells with a cell-permeable dye that is a substrate for  $\beta$ -lactamase. The dye contains two fluorescent moieties. In the intact dye, the two fluorescent moieties are close enough to one another that fluorescent resonance energy transfer (FRET) can take place between them. Following cleavage of the dye into two parts by  $\beta$ -lactamase, the two fluorescent moieties are located on different parts, and thus can drift apart. This increases the distance between the fluorescent moieties, thus decreasing the amount of FRET that can occur between them. It is this decrease in FRET that is measured in the assay.



One skilled in the art can modify the assay described in Zlokarnik and U.S. Patent No. 5,741,657 to form an assay for identifying agonists of GABA<sub>B</sub> receptors by using an inducible promoter to drive  $\beta$ -lactamase that is activated by an intracellular signal generated by the interaction of agonists and the GABA<sub>B</sub> receptor. To produce the GABA<sub>B</sub> receptor, a plasmid encoding HG20 and a plasmid encoding GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b would be transfected into the cells. The cells would be exposed to the cell-permeable dye and then exposed to substances suspected of being agonists of the GABA<sub>B</sub> receptor. Those substances that cause a decrease in FRET are likely to actually be agonists of the GABA<sub>B</sub> receptor.

Accordingly, the present invention includes a method for identifying agonists of the GABA<sub>B</sub> receptor comprising:

- (a) transfecting cells with:
  - (1) an expression vector that directs the expression of HG20 in the cells;
  - (2) an expression vector that directs the expression of GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the cells;
  - (3) an expression vector that directs the expression of  $\beta$ -lactamase under the control of an inducible promoter that is activated by an intracellular signal generated by the interaction of agonists and the GABA<sub>B</sub> receptor;
- (b) exposing the cells to a substrate of  $\beta$ -lactamase that is a cell-permeable dye that contains two fluorescent moieties where the two fluorescent moieties are on different parts of the dye and cleavage of the dye by  $\beta$ -lactamase allows the two fluorescent moieties to drift apart;
- (c) measuring the amount of fluorescent resonance energy transfer (FRET) in the cells in the absence of the substance of step (d);
- (d) exposing the cells to a substance that is suspected of being an agonist of the GABA<sub>B</sub> receptor;
- (e) measuring the amount of FRET in the cells after exposure of the cells to the substance;

wherein if the amount of FRET in the cells measured in step (e) is less than the amount of FRET measured in the cells in step (c), then the substance is an agonist of the GABA<sub>B</sub> receptor.

Substeps (1)-(3) of step (a) can be practiced in any order.

5 The assay described above can be modified to an assay for identifying antagonists of the GABA<sub>B</sub> receptor. Such modification would involve the use of  $\beta$ -lactamase under the control of a promoter that is repressed by at least one intracellular signal generated by interaction of an agonist with the GABA<sub>B</sub> receptor and would also involve running the  
10 assay in the presence of a known agonist. When the cells are exposed to substances suspected of being antagonists of the GABA<sub>B</sub> receptor,  $\beta$ -lactamase will be induced, and FRET will decrease, only if the substance tested is able to counteract the effect of the agonist, i.e., only if the substance tested is actually an antagonist.

15 Accordingly, the present invention includes a method for identifying antagonists of the GABA<sub>B</sub> receptor comprising:

- (a) transfecting cells with:
  - (1) an expression vector that directs the expression of HG20 in the cells;
  - 20 (2) an expression vector that directs the expression of GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the cells;
  - (3) an expression vector that directs the expression of  $\beta$ -lactamase under the control of an inducible promoter that is repressed by at least one intracellular signal generated by interaction of  
25 an agonist with the GABA<sub>B</sub> receptor;
- (b) exposing the cells to a known agonist of the GABA<sub>B</sub> receptor;
- (c) exposing the cells to a substrate of  $\beta$ -lactamase that is a cell-permeable dye that contains two fluorescent moieties where the two  
30 fluorescent moieties are on different parts of the dye and cleavage of the dye by  $\beta$ -lactamase allows the two fluorescent moieties to drift apart;
- (d) measuring the amount of fluorescent resonance energy transfer (FRET) in the cells in the absence of the substance of step (e);

(e) exposing the cells to a substance that is suspected of being an antagonist of the GABA<sub>B</sub> receptor;

(f) measuring the amount of FRET in the cells after exposure of the cells to the substance;

5                    wherein if the amount of FRET in the cells measured in step (f) is less than the amount of FRET measured in the cells in step (d), then the substance is an antagonist of the GABA<sub>B</sub> receptor.

Substeps (1)-(3) of step (a) can be practiced in any order.

10                   In particular embodiments of the assays employing  $\beta$ -lactamase described above, the cells are eukaryotic cells. In particular embodiments, the cells are mammalian cells. In particular embodiments, the cells are selected from the group consisting of: L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92),  
15                   NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, and *Xenopus* oocytes.

20                   In other embodiments, the inducible promoter that is repressed by at least one intracellular signal generated by interaction of an agonist with the GABA<sub>B</sub> receptor is a promoter that is repressed by decreases in cAMP levels or changes in potassium currents.

25                   In other embodiments, the inducible promoter that is activated by at least one intracellular signal generated by interaction of an agonist with the GABA<sub>B</sub> receptor is a promoter that is activated by decreases in cAMP levels or changes in potassium currents.

                    In other embodiments, the known agonist is selected from the group consisting of: GABA, saclofen, (-)baclofen, glycine, and (L)-glutamic acid.

30                   In other embodiments,  $\beta$ -lactamase is TEM-1  $\beta$ -lactamase from *Escherichia coli*.

                    In other embodiments, the substrate of  $\beta$ -lactamase is CCF2/AM (Zlokarnik et al., 1998, Science 279:84-88).

In other embodiments, HG20 has an amino acid sequence of SEQ.ID.NO.:2.

In other embodiments of the above-described methods, HG20 comprises an amino acid sequence selected from the group consisting of:

- 5           SEQ.ID.NO.:2;  
          Positions 9-941 of SEQ.ID.NO.:2;  
          Positions 35-941 of SEQ.ID.NO.:2;  
          Positions 36-941 of SEQ.ID.NO.:2;  
          Positions 38-941 of SEQ.ID.NO.:2;  
10          Positions 39-941 of SEQ.ID.NO.:2;  
          Positions 42-941 of SEQ.ID.NO.:2;  
          Positions 44-941 of SEQ.ID.NO.:2;  
          Positions 46-941 of SEQ.ID.NO.:2;  
          Positions 52-941 of SEQ.ID.NO.:2; and  
15          Positions 57-941 of SEQ.ID.NO.:2.

In other embodiments, GABA<sub>B</sub>R1a is murine GABA<sub>B</sub>R1a and has the amino acid sequence SEQ.ID.NO.:20. In other embodiments, GABA<sub>B</sub>R1a is rat GABA<sub>B</sub>R1a and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In other embodiments, 20 GABA<sub>B</sub>R1b is rat GABA<sub>B</sub>R1b and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In other embodiments, GABA<sub>B</sub>R1a is human GABA<sub>B</sub>R1a and has an amino acid sequence selected from the group consisting of: SEQ.ID.NO.:21 and the protein encoded by SEQ.ID.NO.:23.

25           In particular embodiments, the cells express a promiscuous G-protein, *e.g.*, Gα15 or Gα16.

In particular embodiments, the inducible promoter is a promoter that is activated or repressed by NF-κB or NFAT.

30           The assays described above could be modified to identify inverse agonists. In such assays, one would expect a decrease in β-lactamase activity. Similarly, inverse agonists can be identified by modifying the functional assays that were described previously where those functional assays monitored decreases in cAMP levels. In the case

of assays for inverse agonists, increases in cAMP levels would be observed.

Other transcription-based assays that can be used to identify agonists and antagonists of the GABA<sub>B</sub> receptor rely on the use of green fluorescent proteins or luciferase as reported genes. An example of such an assay comprises:

- (a) transfecting cells with:
  - (1) an expression vector that directs the expression of HG20 in the cells;
  - 10 (2) an expression vector that directs the expression of GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the cells;
  - (3) an expression vector that directs the expression of green fluorescent protein (GFP) under the control of an inducible promoter that is activated by an intracellular signal generated by the interaction of agonists and the GABA<sub>B</sub> receptor;
  - 15 (b) measuring the amount of fluorescence from GFP in the cells;
  - (c) exposing the cells to a substance that is suspected of being an agonist of the GABA<sub>B</sub> receptor;
  - 20 (d) measuring the amount of fluorescence from GFP in the cells that have been exposed to the substance;
- wherein if the amount of fluorescence from GFP in the cells measured in step (b) is less than the amount of fluorescence from GFP measured in the cells in step (d), then the substance is an agonist of the GABA<sub>B</sub> receptor.
- 25

The present invention also includes assays for the identification of agonists or antagonists of GABA<sub>B</sub> receptors that are based upon FRET between a first and a second fluorescent dye where the first dye is bound to one side of the plasma membrane of a cell expressing a heterodimer of HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b and the second dye is free to shuttle from one face of the membrane to the other face in response to changes in membrane potential. In certain embodiments, the first dye is impenetrable to the plasma membrane of the cells and is bound predominately to the extracellular surface of the plasma

membrane. The second dye is trapped within the plasma membrane but is free to diffuse within the membrane. At normal (*i.e.*, negative) resting potentials of the membrane, the second dye is bound predominately to the inner surface of the extracellular face of the plasma membrane, thus placing the second dye in close proximity to the first dye. This close proximity allows for the generation of a large amount of FRET between the two dyes. Following membrane depolarization, the second dye moves from the extracellular face of the membrane to the intracellular face, thus increasing the distance between the dyes. This increased distance results in a decrease in FRET, with a corresponding increase in fluorescent emission derived from the first dye and a corresponding decrease in the fluorescent emission from the second dye. See figure 1 of González & Tsien, 1997, *Chemistry & Biology* 4:269-277. See also González & Tsien, 1995, *Biophys. J.* 69:1272-1280 and U.S. Patent No. 5,661,035.

In certain embodiments, the first dye is a fluorescent lectin or a fluorescent phospholipid that acts as the fluorescent donor. Examples of such a first dye are: a coumarin-labeled phosphatidylethanolamine (*e.g.*, N-(6-chloro-7-hydroxy-2-oxo-2H--1-benzopyran-3-carboxamidoacetyl)-dimyristoylphosphatidyl-ethanolamine) or N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-dipalmitoylphosphatidylethanolamine); a fluorescently-labeled lectin (*e.g.*, fluorescein-labeled wheat germ agglutinin). In certain embodiments, the second dye is an oxonol that acts as the fluorescent acceptor. Examples of such a second dye are: bis(1,3-dialkyl-2-thiobarbiturate)trimethineoxonols (*e.g.*, bis(1,3-dihexyl-2-thiobarbiturate)trimethineoxonol) or pentamethineoxonol analogues (*e.g.*, bis(1,3-dihexyl-2-thiobarbiturate)pentamethineoxonol; or bis(1,3-dibutyl-2-thiobarbiturate)pentamethineoxonol). See González & Tsien, 1997, *Chemistry & Biology* 4:269-277 for methods of synthesizing various dyes suitable for use in the present invention. In certain embodiments, the assay may comprise a natural carotenoid, *e.g.*, astaxanthin, in order to reduce photodynamic damage due to singlet oxygen.

Accordingly, the present invention provides a method of identifying agonists of GABA<sub>B</sub> receptors comprising:

(a) providing test cells comprising:

- (1) an expression vector that directs the expression of HG20 in the cells;
- (2) an expression vector that directs the expression of GABABR1a or GABABR1b in the cells;
- 5 (3) an expression vector that directs the expression of an inwardly rectifying potassium channel;
- (4) a first fluorescent dye, where the first dye is bound to one side of the plasma membrane; and
- (5) a second fluorescent dye, where the second  
10 fluorescent dye is free to shuttle from one face of the plasma membrane to the other face in response to changes in membrane potential;
- (b) exposing the test cells to a substance that is suspected of being an agonist of the GABAB receptor;
- (c) measuring the amount of fluorescence resonance  
15 energy transfer (FRET) in the test cells that have been exposed to the substance;
- (d) comparing the amount of FRET exhibited by the test cells that have been exposed to the substance with the amount of FRET exhibited by control cells;
- 20 wherein if the amount of FRET exhibited by the test cells is less than the amount of FRET exhibited by the control cells, the substance is an agonist of the GABAB receptor;
- where the control cells are either (1) cells that are essentially the same as the test cells except that they do not comprise at least one of  
25 the items listed at (a) (1)-(5) but have been exposed to the substance; or (2) test cells that have not been exposed to the substance.
- The above-described assay can be easily modified to form a method to identify antagonists of the GABAB receptor. Such a method comprises:
- 30 (a) providing test cells comprising:
  - (1) an expression vector that directs the expression of HG20 in the cells;
  - (2) an expression vector that directs the expression of GABABR1a or GABABR1b in the cells;

(3) an expression vector that directs the expression of an inwardly rectifying potassium channel;

(4) a first fluorescent dye, where the first dye is bound to one side of the plasma membrane; and

5 (5) a second fluorescent dye, where the second fluorescent dye is free to shuttle from one face of the plasma membrane to the other face in response to changes in membrane potential;

(b) exposing the test cells to a known agonist of the GABA<sub>B</sub> receptor in the presence of a substance that is suspected of being  
10 an antagonist of the GABA<sub>B</sub> receptor;

(c) exposing the test cells to the known agonist of the GABA<sub>B</sub> receptor in the absence of the substance that is suspected of being an antagonist of the GABA<sub>B</sub> receptor;

(d) measuring the amount of fluorescence resonance  
15 energy transfer (FRET) in the test cells of steps (b) and (c);

(e) comparing the amount of FRET exhibited by the test cells of steps (b) and (c);

where if the amount of FRET exhibited by the test cells of step (b) is greater than the amount of FRET exhibited by the test cells of  
20 step (c), the substance is an antagonist of the GABA<sub>B</sub> receptor.

In particular embodiments of the above-described methods, the expression vectors are transfected into the test cells.

In particular embodiments of the above-described methods, HG20 has an amino acid sequence of SEQ.ID.NO.:2.

25 In particular embodiments of the above-described methods, HG20 comprises an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;

Positions 9-941 of SEQ.ID.NO.:2;

30 Positions 35-941 of SEQ.ID.NO.:2;

Positions 36-941 of SEQ.ID.NO.:2;

Positions 38-941 of SEQ.ID.NO.:2;

Positions 39-941 of SEQ.ID.NO.:2;

Positions 42-941 of SEQ.ID.NO.:2;



Positions 44-941 of SEQ.ID.NO.:2;  
Positions 46-941 of SEQ.ID.NO.:2;  
Positions 52-941 of SEQ.ID.NO.:2; and  
Positions 57-941 of SEQ.ID.NO.:2.

5           In particular embodiments of the above-described methods,  
GABABR1a is murine GABABR1a and has the amino acid sequence  
SEQ.ID.NO.:20. In particular embodiments, GABABR1a is rat  
GABABR1a and has the amino acid sequence reported in Kaupmann et  
al., 1997, Nature 386:239-246. In particular embodiments, GABABR1b is  
10   rat GABABR1b and has the amino acid sequence reported in Kaupmann  
et al., 1997, Nature 386:239-246. In particular embodiments, GABABR1a  
is human GABABR1a and has an amino acid sequence selected from the  
group consisting of: SEQ.ID.NO.:21 and the protein encoded by  
SEQ.ID.NO.:23.

15           Inwardly rectifying potassium channels that are suitable for  
use in the methods of the present invention are disclosed in, *e.g.*, Misgeld  
et al., 1995, Prog. Neurobiol. 46:423-462; North, 1989, Br. J. Pharmacol.  
98:13-23; Gahwiler et al., 1985, Proc. Natl. Acad. Sci USA 82:1558-1562;  
Andrade et al., 1986, Science 234:1261.

20           In particular embodiments of the above-described methods,  
the first fluorescent dye is selected from the group consisting of: a  
fluorescent lectin; a fluorescent phospholipid; a coumarin-labeled  
phosphatidylethanolamine; N-(6-chloro-7-hydroxy-2-oxo-2H--1-  
benzopyran-3-carboxamidoacetyl)-dimyristoylphosphatidyl-ethanolamine);  
25   N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-  
dipalmitoylphosphatidylethanolamine); and fluorescein-labeled wheat  
germ agglutinin.

          In particular embodiments of the above-described methods,  
the second fluorescent dye is selected from the group consisting of: an  
30   oxonol that acts as the fluorescent acceptor; bis(1,3-dialkyl-2-  
thiobarbiturate)trimethineoxonols; bis(1,3-dihexyl-2-  
thiobarbiturate)trimethineoxonol; bis(1,3-dialkyl-2-  
thiobarbiturate)quatramethineoxonols; bis(1,3-dialkyl-2-  
thiobarbiturate)pentamethineoxonols; bis(1,3-dihexyl-2-

thiobarbiturate)pentamethineoxonol; bis(1,3-dibutyl-2-thiobarbiturate)pentamethineoxonol); and bis(1,3-dialkyl-2-thiobarbiturate)hexamethineoxonols.

In a particular embodiment of the above-described methods, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK-) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* melanophores, or *Xenopus* oocytes.

In a particular embodiment of the above-described methods, the cells are transfected with separate expression vectors that direct the expression of HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the cells. In other embodiments, the cells are transfected with a single expression vector that direct the expression of both HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the cells.

The conditions under which step (b) of the first method described above and steps (b) and (c) of the second method described above are practiced are conditions that are typically used in the art for the study of protein-ligand interactions: e.g., physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

The GABA<sub>B</sub> receptor belongs to the class of proteins known as G-protein coupled receptors (GPCRs). GPCRs transmit signals across cell membranes upon the binding of ligand. The ligand-bound GPCR interacts with a heterotrimeric G-protein, causing the G $\alpha$  subunit of the G-protein to disassociate from the G $\beta$  and G $\gamma$  subunits. The G $\alpha$  subunit can then go on to activate a variety of second messenger systems.

Generally, a particular GPCR is only coupled to a particular type of G-protein. Thus, to observe a functional response from the GPCR, it is necessary to ensure that the proper G-protein is present in the system containing the GPCR. It has been found, however, that there are certain

G-proteins that are "promiscuous." These promiscuous G-proteins will couple to, and thus transduce a functional signal from, virtually any GPCR. See Offermanns & Simon, 1995, J. Biol. Chem. 270:15175, 15180 (Offermanns). Offermanns described a system in which cells are  
5 transfected with expression vectors that result in the expression of one of a large number of GPCRs as well as the expression of one of the promiscuous G-proteins  $G\alpha 15$  or  $G\alpha 16$ . Upon the addition of an agonist of the GPCR to the transfected cells, the GPCR was activated and was able, via  $G\alpha 15$  or  $G\alpha 16$ , to activate the  $\beta$  isoform of phospholipase C, leading to  
10 an increase in inositol phosphate levels in the cells.

Therefore, by making use of these promiscuous G-proteins as in Offermanns, it is possible to set up functional assays for the GABA<sub>B</sub> receptor, even in the absence of knowledge of the G-protein with which the GABA<sub>B</sub> receptor is coupled *in vivo*. One possibility for utilizing  
15 promiscuous G-proteins in connection with the GABA<sub>B</sub> receptor includes a method of identifying agonists of the GABA<sub>B</sub> receptor comprising:

- (a) providing cells that express HG20, GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, and a promiscuous G-protein, where HG20 and either GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b form a heterodimer representing a functional  
20 GABA<sub>B</sub> receptor;
- (b) exposing the cells to a substance that is a suspected agonist of the GABA<sub>B</sub> receptor;
- (c) measuring the level of inositol phosphates in the cells; where an increase in the level of inositol phosphates in the  
25 cells as compared to the level of inositol phosphates in the cells in the absence of the suspected agonist indicates that the substance is an agonist of the GABA<sub>B</sub> receptor.

Levels of inositol phosphates can be measured by monitoring calcium mobilization. Intracellular calcium mobilization is typically  
30 assayed in whole cells under a microscope using fluorescent dyes or in cell suspensions via luminescence using the aequorin assay.

In methods related to those described above, rather than using changes in inositol phosphate levels as an indication of GABA<sub>B</sub> receptor function, potassium currents are measured. This is feasible since

the GABA<sub>B</sub> receptor, like other metabotropic receptors, is expected to be coupled to potassium channels. Thus, one could measure GABA<sub>B</sub> receptor coupling to GIRK2 channels or to other potassium channels in oocytes.

In a particular embodiment of the above-described method,  
5 the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92),  
10 NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), or Xenopus oocytes.

In a particular embodiment of the above-described method,  
15 the cells are transfected with expression vectors that direct the expression of HG20, GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, and the promiscuous G-protein in the cells.

The conditions under which step (b) of the method is practiced are conditions that are typically used in the art for the study of protein-ligand interactions: *e.g.*, physiological pH; salt conditions such as  
20 those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In a particular embodiment of the above-described method, the promiscuous G-protein is selected from the group consisting of Gα15 or Gα16. Expression vectors containing Gα15 or Gα16 are known in the  
25 art. See, *e.g.*, Offermanns; Buhl et al., 1993, FEBS Lett. 323:132-134; Amatruda et al., 1993, J. Biol. Chem. 268:10139-10144.

The above-described assay can be easily modified to form a method to identify antagonists of the GABA<sub>B</sub> receptor. Such a method is also part of the present invention and comprises:

- 30 (a) providing cells that express HG20, GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, and a promiscuous G-protein;
- (b) exposing the cells to a substance that is an agonist of the GABA<sub>B</sub> receptor;

(c) subsequently or concurrently to step (b), exposing the cells to a substance that is a suspected antagonist of the GABA<sub>B</sub> receptor;  
(d) measuring the level of inositol phosphates in the cells; where a decrease in the level of inositol phosphates in the cells in the presence of the suspected antagonist as compared to the level of inositol phosphates in the cells in the absence of the suspected antagonist indicates that the substance is an antagonist of the GABA<sub>B</sub> receptor.

In a particular embodiment of the above-described method, the agonist is an amino acid such as GABA, glutamate, glycine, or amino acid analogues such as (-)baclofen.

In a particular embodiment of the above-described method, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other embodiments, the cells are L cells L-M(TK-) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), or Xenopus oocytes.

The conditions under which steps (b) and (c) of the method are practiced are conditions that are typically used in the art for the study of protein-ligand interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In a particular embodiment of the above-described method, the cells are transfected with expression vectors that direct the expression of HG20, GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b, and the promiscuous G-protein in the cells.

In a particular embodiment of the above-described method, the promiscuous G-protein is selected from the group consisting of Gα15 or Gα16.

In particular embodiments of the above-described methods, HG20 has an amino acid sequence of SEQ.ID.NO.:2.

In other embodiments of the above-described methods, HG20 comprises an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;

Positions 9-941 of SEQ.ID.NO.:2;

5 Positions 35-941 of SEQ.ID.NO.:2;

Positions 36-941 of SEQ.ID.NO.:2;

Positions 38-941 of SEQ.ID.NO.:2;

Positions 39-941 of SEQ.ID.NO.:2;

Positions 42-941 of SEQ.ID.NO.:2;

10 Positions 44-941 of SEQ.ID.NO.:2;

Positions 46-941 of SEQ.ID.NO.:2;

Positions 52-941 of SEQ.ID.NO.:2; and

Positions 57-941 of SEQ.ID.NO.:2.

In other embodiments, GABA<sub>B</sub>R1a is murine GABA<sub>B</sub>R1a  
15 and has the amino acid sequence SEQ.ID.NO.:20. In other embodiments, GABA<sub>B</sub>R1a is rat GABA<sub>B</sub>R1a and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In other embodiments, GABA<sub>B</sub>R1b is rat GABA<sub>B</sub>R1b and has the amino acid sequence reported in Kaupmann et al., 1997, Nature 386:239-246. In other embodiments,  
20 GABA<sub>B</sub>R1a is human GABA<sub>B</sub>R1a and has an amino acid sequence selected from the group consisting of: SEQ.ID.NO.:21 and the protein encoded by SEQ.ID.NO.:23.

While the above-described methods are explicitly directed to testing whether "a" substance is an agonist or antagonist of the GABA<sub>B</sub>  
25 receptor, it will be clear to one skilled in the art that such methods can be adapted to test collections of substances, e.g., combinatorial libraries, to determine whether any members of such collections are activators or inhibitors of the GABA<sub>B</sub> receptor. Accordingly, the use of collections of substances, or individual members of such collections, as the substance in  
30 the above-described methods is within the scope of the present invention.

The present invention includes pharmaceutical compositions comprising agonists and antagonists of GABA<sub>B</sub> receptors that have been identified by the above-described methods. The agonists and antagonists are generally combined with pharmaceutically acceptable carriers to form

pharmaceutical compositions. Examples of such carriers and methods of formulation of pharmaceutical compositions containing agonists and antagonists and carriers can be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain a therapeutically effective amount of the agonists and antagonists.

Therapeutic or prophylactic compositions are administered to an individual in amounts sufficient to treat or prevent conditions where GABA<sub>B</sub> receptor activity is abnormal. The effective amount can vary according to a variety of factors such as the individual's condition, weight, gender, and age. Other factors include the mode of administration. The appropriate amount can be determined by a skilled physician.

Compositions can be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents can be desirable.

The compositions can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compositions can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they can also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

Advantageously, compositions can be administered in a single daily dose, or the total daily dosage can be administered in divided doses of two, three or four times daily. Furthermore, compositions can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

The dosage regimen utilizing the compositions is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal, hepatic and cardiovascular function of the patient; and the particular composition thereof employed. A physician of ordinary skill can readily determine and prescribe the effective amount of the composition required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of composition within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the composition's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a composition.

Agonists and antagonists identified by the above-described methods are useful in the same manner as well-known agonists and antagonists of other GABA<sub>B</sub> receptors. For example, (-) baclofen is a known agonist of GABA<sub>B</sub> receptors and, in racemic form, is a clinically useful muscle relaxant known as LIORESAL® (Bowery & Pratt, 1992, *Arzneim.-Forsch./Drug Res.* 42:215-223 [Bowery & Pratt]). Similarly, the agonists and antagonists of GABA<sub>B</sub> receptors identified by the methods of the present invention are expected to be useful as muscle relaxants. Bowery & Pratt, at Table 1, page 219, list the therapeutic potential of GABA<sub>B</sub> receptor agonists and antagonists. For agonists, the therapeutic potential is said to include use as muscle relaxants and anti-asthmatics. For antagonists, the therapeutic potential is said to include use as antidepressants, anticonvulsants, nootropics, and anxiolytics. Additionally, at page 220, left column, Bowery & Pratt list some additional therapeutic uses for the GABA<sub>B</sub> receptor agonist (-) baclofen: treatment of trigeminal neuralgia and reversal of ethanol withdrawal symptoms. Given the wide range of utility displayed by known agonists and antagonists of GABA<sub>B</sub> receptors, it is clear that those skilled in the art would consider the agonists and antagonists identified by the methods of the present invention to be pharmacologically useful. In addition, it is believed that such agonists and antagonists will also be useful in the treatment of epilepsy, neuropsychiatric disorders, and dementias.



When screening compounds in order to identify potential pharmaceuticals that specifically interact with a target receptor, it is necessary to ensure that the compounds identified are as specific as possible for the target receptor. To do this, it is necessary to screen the compounds against as wide an array as possible of receptors that are similar to the target receptor. Thus, in order to find compounds that are potential pharmaceuticals that interact with receptor A, it is necessary not only to ensure that the compounds interact with receptor A (the "plus target") and produce the desired pharmacological effect through receptor A, it is also necessary to determine that the compounds do not interact with receptors B, C, D, etc (the "minus targets"). In general, as part of a screening program, it is important to have as many minus targets as possible (see Hodgson, 1992, Bio/Technology 10:973-980, at 980). HG20 protein, DNA encoding HG20 protein, GABABR1a protein, DNA encoding GABABR1a protein, and recombinant cells that have been engineered to express HG20 protein and GABABR1a protein have utility in that they can be used as "minus targets" in screens design to identify compounds that specifically interact with other G-protein coupled receptors, *i.e.*, non-GABAB receptors.

The present invention also includes antibodies to the HG20 protein. Such antibodies may be polyclonal antibodies or monoclonal antibodies. The antibodies of the present invention are raised against the entire HG20 protein or against suitable antigenic fragments of the protein that are coupled to suitable carriers, *e.g.*, serum albumin or keyhole limpet hemocyanin, by methods well known in the art. Methods of identifying suitable antigenic fragments of a protein are known in the art. See, *e.g.*, Hopp & Woods, 1981, Proc. Natl. Acad. Sci. USA 78:3824-3828; and Jameson & Wolf, 1988, CABIOS (Computer Applications in the Biosciences) 4:181-186. Particularly suitable peptides are: amino acids 357-371 of SEQ.ID.NO.:2 and amino acids 495-511 of SEQ.ID.NO.:2. Also, anti-peptide antisera can be generated by immunization of New Zealand White rabbits with a KLH-conjugation of a 20 amino acid synthetic peptide corresponding to residues 283-302 of HG20 (GWYEPSWWEQVHTEANSSRC) (a portion of SEQ.ID.NO.:2).

For the production of polyclonal antibodies, HG20 protein or an antigenic fragment, coupled to a suitable carrier, is injected on a periodic basis into an appropriate non-human host animal such as, *e.g.*, rabbits, sheep, goats, rats, mice. The animals are bled periodically and sera obtained are tested for the presence of antibodies to the injected antigen. The injections can be intramuscular, intraperitoneal, subcutaneous, and the like, and can be accompanied with adjuvant.

For the production of monoclonal antibodies, HG20 protein or an antigenic fragment, coupled to a suitable carrier, is injected into an appropriate non-human host animal as above for the production of polyclonal antibodies. In the case of monoclonal antibodies, the animal is generally a mouse. The animal's spleen cells are then immortalized, often by fusion with a myeloma cell, as described in Kohler & Milstein, 1975, *Nature* 256:495-497. For a fuller description of the production of monoclonal antibodies, see Antibodies: A Laboratory Manual, Harlow & Lane, eds., Cold Spring Harbor Laboratory Press, 1988.

Gene therapy may be used to introduce HG20 polypeptides into the cells of target organs. Nucleotides encoding HG20 polypeptides can be ligated into viral vectors which mediate transfer of the nucleotides by infection of recipient cells. Suitable viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, and polio virus based vectors. Alternatively, nucleotides encoding HG20 polypeptides can be transferred into cells for gene therapy by non-viral techniques including receptor-mediated targeted transfer using ligand-nucleotide conjugates, lipofection, membrane fusion, or direct microinjection. These procedures and variations thereof are suitable for *ex vivo* as well as *in vivo* gene therapy. Gene therapy with HG20 polypeptides will be particularly useful for the treatment of diseases where it is beneficial to elevate HG20 activity.

The following non-limiting examples are presented to better illustrate the invention.

## EXAMPLE 1

Cloning and sequencing of HG20

A cDNA fragment encoding full-length HG20 can be isolated from a human fetal brain cDNA library by using the polymerase chain  
5 reaction (PCR) employing the following primer pair:

HG20.F139 5'-CCGTTCTGAGCCGAGCCG -3' (SEQ.ID.NO.:3)

HG20.R3195 5'-TCCGCAGCCAGAGCCGACAG -3' (SEQ.ID.NO.:4)

10 The above primer pair is meant to be illustrative only. Those skilled in the art would recognize that a large number of primer pairs, based upon SEQ.ID.NO.:1, could also be used.

PCR reactions can be carried out with a variety of thermostable enzymes including but not limited to AmpliTaq, AmpliTaq  
15 Gold, Vent polymerase. For AmpliTaq, reactions can be carried out in 10 mM Tris-Cl, pH 8.3, 2.0 mM MgCl<sub>2</sub>, 200 μM for each dNTP, 50 mM KCl, 0.2 μM for each primer, 10 ng of DNA template, 0.05 units/μl of AmpliTaq. The reactions are heated at 95°C for 3 minutes and then cycled 35 times using the cycling parameters of 95°C, 20 seconds, 62°C, 20 seconds, 72°C,  
20 3 minutes. In addition to these conditions, a variety of suitable PCR protocols can be found in PCR Primer, A Laboratory Manual, edited by C.W. Dieffenbach and G.S. Dveksler, 1995, Cold Spring Harbor Laboratory Press.

A suitable cDNA library from which a clone encoding HG20  
25 can be isolated would be a random primed fetal brain cDNA library consisting of approximately 4.0 million primary clones constructed in the plasmid vector pBluescript (Stratagene, LaJolla, CA). The primary clones of such a library can be subdivided into pools with each pool containing approximately 20,000 clones and each pool can be amplified separately.

30 By this method, a cDNA fragment (SEQ.ID.NO.:1) encoding an open reading frame of 941 amino acids (SEQ.ID.NO.:2) is obtained. This cDNA fragment can be cloned into a suitable cloning vector or

expression vector. For example, the fragment can be cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, San Diego, CA). HG20 protein can then be produced by transferring an expression vector containing SEQ.ID.NO.:1 or portions thereof into a suitable host cell and  
 5 growing the host cell under appropriate conditions. HG20 protein can then be isolated by methods well known in the art.

Alternatively, other cDNA libraries made from human tissues that express HG20 RNA can be used with PCR primers HG20.F139 and HG20.R3195 in order to amplify a cDNA fragment  
 10 encoding full-length HG20. Suitable cDNA libraries would be those prepared from cortex, cerebellum, testis, ovary, adrenal gland, thyroid, or spinal cord.

As an alternative to the above-described PCR method, a cDNA clone encoding HG20 can be isolated from a cDNA library using as  
 15 a probe oligonucleotides specific for HG20 and methods well known in the art for screening cDNA libraries with oligonucleotide probes. Such methods are described in, e.g., Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, *DNA Cloning: A Practical Approach*, MRL Press, Ltd., Oxford, U.K., Vol. I, II. Oligonucleotides that  
 20 are specific for HG20 and that can be used to screen cDNA libraries are:

	HG20.F46	5'-GGGATGATCATGGCCAGTGC-3' (SEQ.ID.NO.:5)
	HG20.R179	5'-GGATCCATCAAGGCCAAAGA-3' (SEQ.ID.NO.:6)
25	HG21.F43	5'-GCCGCTGTCTCCTTCCTGA-3' (SEQ.ID.NO.:7)
	HG21.R251	5'-TTGGTTCACACTGGTGACCGA-3' (SEQ.ID.NO.:8)
	HG20.R123	5'-TTCACCTCCCTGCTGTCTTG-3' (SEQ.ID.NO.:9)
	HG20.F1100	5'-CAGGCGATTCCAGTTCACTCA-5' (SEQ.ID.NO.:10)
	HG20.F1747	5'-GAACCAAGCCAGCACATCCC-3' (SEQ.ID.NO.:11)
30	HG20.R54	5'-CCTCGCCATACAGAACTCC-3' (SEQ.ID.NO.:12)
	HG20.R75	5'-GTGTCATAGAGCCGAGGTC-3' (SEQ.ID.NO.:13)
	HG20.F139	5'-CCGTTCTGAGCCGAGCCG-3' (SEQ.ID.NO.:3)
	HG20.R3195	5'-TCCGCAGCCAGAGCCGACAG-3' (SEQ.ID.NO.:4)

Membrane-spanning proteins, such as GABAB receptors, when first translated generally possess an approximately 16 to 40 amino acid segment known as a signal sequence. Signal sequences direct the nascent protein to be transported through the endoplasmic reticulum membrane, following which signal sequences are cleaved from the protein. Signal sequences generally contain from 4 to 12 hydrophobic residues but otherwise possess little sequence homology. The Protein Analysis tool of the GCG program (Genetics Computer Group, Madison, Wisconsin), a computer program capable of identifying likely signal sequences, was used to examine the N terminus of HG20. Several likely candidates for cleavage sites which would generate mature HG20 protein, *i.e.*, protein lacking the signal sequence, were identified. The results are shown in Figure 3.

## EXAMPLE 2

### Expression of HG20 in normal and diseased adrenal tissue

Northern blots were performed to measure the amount of HG20 RNA in normal and diseased adrenal tissue. The results are shown in Table 2 below. The amount of the approximately 6.5 kb HG20 transcript is shown normalized to the amount of  $\beta$ -actin transcript.

Table 2

<u>Pathology</u>	<u>Profile</u>	<u>HG20</u> <u>RNA</u>	<u>Actin</u> <u>RNA</u>	<u>HG20</u> <u>/actin</u>
Pheochromocytoma	M, 30 yr	0.47	0.74	0.64
Adrenal carcinoma cortex	M, 69 yr	0.61	0.80	0.76
Adrenal adenoma cortex	M, 69 yr	0.62	1.15	0.54
Normal Adrenal	M, 26 yr	1.00	1.00	1.00

The results shown in Table 2 indicate that HG20 expression is decreased in diseased states of the adrenal gland. Thus, increasing the

concentration of HG20 in such diseased states is likely to be pharmacologically useful. Accordingly, one skilled in the art would expect agonists of HG20 to be pharmacologically useful.

5

## EXAMPLE 3

Tissue distribution of various HG20 RNA transcripts

Table 3, below, shows the results of experiments to measure the amount of HG20 RNA transcripts of various lengths in various tissues. The results shown were derived from a multiple tissue Northern blot that was hybridized overnight in expressHyb solution (Clontech).  
 10 Washing conditions were: 0.1X SSC, 0.1% SDS, at 60°C.  
 A <sup>32</sup>P-random primer labelled Eco RI fragment containing the full-length native HG20 DNA was used as a hybridization probe. The greater the number of plus signs in a particular tissue, the greater was the amount of  
 15 HG20 RNA detected in that tissue.

Table 3

Tissue	6.5 kb	4.5 kb	4.0 kb	1.8 kb
cerebellum	++	+		
cerebral cortex	++++	+		
medulla	+	+		
occipital pole	+	+		
frontal lobe	+++	+		
temporal lobe	+++	+		
putamen	++	+		
spinal cord n=2	++	+		
amygdala	+++			
caudate nucleus	+	+		
corpus callosum	+	+		
hippocampus	++	+		
whole brain	+++	+		
substantia nigra	+	+		
subthalamie nucleus	+	+		
thalamus	++	+		

spleen		+		
thymus n=2		++		
prostate		++		
testis n=2	++	+	+++	
ovary		++	+	+
small intestine n=2		++		
colon (mucosal lining)		++		
peripheral blood leucocytes		++		
stomach n=2	+	+		
thyroid n=2	++	++++		
lymph node		+		
trachea		++		
adrenal gland	+++	+++	+	++++
bone marrow		++		
heart	+	++		
brain	+++++			
placenta		+		
lung		+		
liver		+		
skeletal muscle	+	++		
kidney		+		
pancreas	+	+		
adrenal medulla	+++			+
adrenal cortex	+++++		++	++

The distribution of HG20 RNA shown in Table 3 suggests that HG20 mediates activities of the central and peripheral nervous system.

5

#### EXAMPLE 4

##### Distribution of HG20 mRNA in brain

Using in situ hybridisation, the distribution of HG20 mRNA in squirrel monkey brain was studied. Antisense oligonucleotide probes to HG20 were generated on an Applied Biosystems Model 394 DNA synthesiser and purified by preparative polyacrylamide electrophoresis.

10

Probe 1: 5'ATC-TGG-GTT-TGT-TCT-CAG-GGT-GAT-GAG-CTT-CGG-CAC-GAA-TAC-CAG 3' (SEQ.ID.NO.:14);

Probe2: 5' GCT-CTG-TGA-TCT-TCA-TTC-GCA-GGC-GAT-GGT-TTT-CTG-ACT-GTA-GGC 3' (SEQ.ID.NO.:15).

- 5 Each oligonucleotide was 3'-end labelled with [<sup>35</sup>S] deoxyadenosine 5'-(thiotriphosphate) in a 30:1 molar ratio of <sup>35</sup>S-isotope:oligonucleotide using terminal deoxynucleotidyl transferase for 15 min at 37°C in the reaction buffer supplied (Boehringer). Radiolabelled oligonucleotide was separated from unincorporated nucleotides using Sephadex G50 spin
- 10 columns. The specific activities of the labelled probes in several labelling reactions varied from 1.2-2.3 x 10<sup>9</sup> cpm/mg. Squirrel monkey brains were removed and fresh frozen in 1 cm blocks. 12 mm sections were taken and fixed for *in situ* hybridisation. Hybridisation of the sections was carried out according to the method of Sirinathsinghji et al., 1993, Neuroreports
- 15 4:175-178. Briefly, sections were removed from alcohol, air dried and 5 x10<sup>5</sup> cpm of each <sup>35</sup>S-labelled probe (both oligonucleotides) in 100 ml of hybridisation buffer was applied to each slide. Labelled "antisense" probe was also used in the presence of an excess (100x) concentration of unlabelled antisense probe to define non-specific hybridisation. Parafilm
- 20 coverslips were placed over the sections which were incubated overnight (about 16 hr) at 37°C. Following hybridisation the sections were washed for 1 hr at 57°C in 1xSSC, then rinsed briefly in 0.1xSSC, dehydrated in a series of alcohols, air dried, and exposed to Amersham Hyperfilm bmax X-ray film. Autoradiographs were analysed using a MCID computerised
- 25 image analysis system (Image Research Inc., Ontario, Canada).

Highest levels of mRNA for HG20 were found in the hippocampus (dentate gyrus, CA3, CA2, and CA1). High levels were also seen in cortical regions (frontal, cingulate, temporal parietal, entorhinal, and visual) and the cerebellum, although medial septum, thalamic nuclei

30 (medial-dorsal and lateral posterior), lateral geniculates, red nucleus, reticular formation, and griseum pontis all show expression of message. While there are many similarities with the distribution reported for the GABA<sub>B</sub> receptor mRNA in rat, one marked difference is that expression of HG20 mRNA in the monkey caudate and putamen is below the level of



detection while cortical levels are high. In the rat, the GABA<sub>B</sub> receptor mRNA appears equally expressed in striatum as in cortex. Figure 4 illustrates these results.

5

## EXAMPLE 5

### Attempted recombinant expression of full-length HG20 protein

Following the cloning of HG20 DNA, attempts were made to express full-length HG20 protein (941 amino acids) using various eukaryotic cell lines and expression vectors. The cell lines that were used were: COS-7 cells, HEK293 cells, and frog melanophores. The expression vectors that were used to attempt to express the full-length HG20 protein were: pCR3.1 and pcDNA3.1 (Invitrogen, San Diego, CA) and pcINEO (promega)

All of the attempts to express full-length HG20 described above were unsuccessful. See, *e.g.*, Figure 7, second bar from the left, marked "HG20." See also Figure 5A, lane 1. Although the reason for these failures is not known, it is possible that the highly GC rich nature of the region of the HG20 mRNA that encodes amino acids 1-51 results in the formation of secondary structure in the mRNA that impedes translation. It was only after the construction of an expression vector that encodes a truncated HG20 protein, lacking the first 51 amino acids, that HG20 was successfully expressed. Figure 5A-B shows the results of the successful expression of an HG20 protein having amino acids 52-941. It is expected that expression of HG20 proteins having amino acids 53-941, 54-941, 55-941, *etc.*, could be accomplished in a similar manner. It is also expected that expression of HG20 proteins having the above-described amino termini but having different carboxyl termini could be accomplished in a similar manner as well. Thus, the expression of an HG20 protein having an amino terminus as listed above and having a truncated carboxyl terminus could be accomplished. Alternatively, the carboxyl terminus could be fused to non-HG20 amino acid sequences, forming a chimeric HG20 protein. It is also possible to express HG20

having an amino terminus listed above as a chimeric protein with non-HG20 sequences fused to the amino terminus.

Figure 5A shows the expression of amino acids 52-941 of HG20 as part of a chimeric or fusion protein with the FLAG epitope fused to the amino terminus of the HG20 sequences in a coupled *in vitro* transcription/translation experiment. Figure 5B shows the expression of amino acids 52-941 of HG20 as part of a chimeric or fusion protein with the FLAG epitope fused to the amino terminus of the HG20 sequences in COS-7 cells and melanophores. The expression vector used in this experiment was pcDNA3.1. The expression constructs used in Figure 5A-B also encoded a cleavable signal sequence from the influenza hemagglutinin gene that has been shown to facilitate the membrane insertion of G-protein coupled receptors (Guan et al., 1992, J. Biol. Chem. 267:21995-21998) and the fusion proteins were detected with anti-FLAG antibody. The expression constructs had also been engineered to contain a Kozak consensus sequence prior to the initiating ATG. The amino acid sequences of the hemagglutinin signal sequence and the FLAG epitope were:

[MKTIIALSYIFCLVFA] [DYKDDDDK] SEQ.ID.NO:17  
 HA signal peptide      FLAG epitope

Amino acids 57-941 have been expressed in mammalian cells as part of a chimeric protein. A chimeric construct of HG20 was made that consisted of bases -224 to 99 of the bovine GABA<sub>A</sub>  $\alpha$ 1 gene, a sequence encoding the c-myc epitope tag (amino acid residues 410-419 of the human oncogene product c-myc), a cloning site encoding the amino acid asparagine, and DNA encoding residues 57-941 of HG20. The resultant chimeric protein has the amino acid sequence shown below, with the construct cloned into pcDNA1.1Amp (Invitrogen, San Diego, CA).

\_\_\_\_Bovine alpha 1 signal seq\_\_\_\_c-myc\_\_\_\_\_  
 MKKSPGLSDYLWAWTLFLSTLTGRSYGQPSLQD EQKLISEEDL N  
 \_res. 57-941 HG20\_

SIMGLMPLT... (SEQ.ID.NO.:18)

The three periods "..." indicate that the chimeric protein sequence extends until amino acid 941 of HG20.

5           The cell surface expression of this construct was verified using a cell surface ELISA technique. Briefly, HEK293 cells were seeded at ~1x10<sup>5</sup> cells per well in a 24 well tissue culture plate and allowed to adhere for 24 hours. Each well was transfected with a total of 1 µg of DNA. In addition to tagged and un-tagged HG20 constructs, c-myc tagged  
10   GABA<sub>A</sub> α1 was transfected with GABA<sub>A</sub> β1 as a positive control for cell surface expression. Two days after transfection, the cells were assayed for surface expression of the c-myc epitope using the 9E10 monoclonal antibody raised to the c-myc epitope, followed by HRP (horse radish peroxidase) conjugated anti-mouse antibody (Promega) and colormetric  
15   development using K-Blue (Bionostics). The results are shown in Figure 7. Figure 7 demonstrates that when HG20 is part of a chimeric protein, it can be expressed well in mammalian cells but that when attempts are made to express full-length HG20 (amino acids 1-941) directly, *i.e.*, not as part of a chimeric protein, essentially no expression is observed.

20

#### EXAMPLE 6

##### Construction of Full Length Murine GABA<sub>B</sub>R1a Coding Region

          Using a combination of TFASTX (Pearson et al., 1997, Genomics 46:24-36) and TBLASTX (Altschul et al., 1997, Nucleic Acids  
25   Res. 25:3389-3402) searching programs against dbEST: Database of Expressed Sequence Tags (URL <http://www.ncbi.nlm.nih.gov/dbEST/index.html>), we identified partial cDNA clones in the EST collection which encoded murine GABA<sub>B</sub>R1a using the rat GABA<sub>B</sub> receptor subunit  
cDNAs (GenBank Accession Numbers Y10369 and Y10370) as probe  
30   sequences (Kaupmann et al., 1997, Nature 386:239-246). Two of these ESTs (IMAGE Consortium clone identification numbers 472408 and 319196) were obtained (Research Genetics, Birmingham, Ala). The DNA

sequences of both cDNA clones were determined using standard methods on an ABI 373a automated sequencer (Perkin-Elmer-Applied Biosystems, Foster City, CA).

The partial cDNAs were assembled by long accurate PCR using the following oligonucleotides: 472408 sense: 5' - GC GAATTC GGTACC ATG CTG CTG CTG CTG CTG GTG CCT - 3' (SEQ.ID.NO.:24), 472408 antisense: 5' - GG GAATTC TGG ATA TAA CGA GCG TGG GAG TTG TAG ATG TTA AA - 3' (SEQ.ID.NO.:25), 319196 sense: 5' - CCA GAATTC CCA GCC CAA CCT GAA CAA TC - 3' (SEQ.ID.NO.:26), 319196 antisense: 5' - CG GCGGCCGC TCA CTT GTA AAG CAA ATG TA - 3' (SEQ.ID.NO.:27) which amplified two fragments corresponding to the 5' 2,100 basepairs and 3' 1,000 basepairs of the murine GABAR1a coding region. The PCR conditions were 200 ng of cDNA template, 2.5 units of Takara LA Taq (PanVera, Madison, WI), 25 mM TAPS (pH 9.3), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 1 mM 2-mercaptoethanol, 100 mM each dNTP and 1 mM each primer with cycling as follows 94°C 1 min, 9 cycles of 98°C for 20 seconds, 72°C-56°C (decreases 2°C per cycle), 72°C for 30 seconds, followed by 30 cycles of 98°C for 20 seconds, 60°C for 3 minutes. A final extension at 72°C for 10 minutes was performed. PCR products were cloned into the TA-Cloning vector pCRII-TOPO (Invitrogen, San Diego, CA) following the manufacturers directions. Cloned PCR products were confirmed by DNA sequencing. To form full-length cDNA, the pCINeo mammalian expression vector was digested with EcoRI and NotI. The EcoRI fragment from PCR cloning of 472408 and the EcoRI/NotI product from PCR cloning of 319196 were ligated in a three part ligation with digested pCINeo vector. The resulting clones were screened by restriction digestion with SstI which cuts once in the vector and once in the 472408 derived fragment. The resulting expression clone is 2,903 basepairs in length. The overall cDNA length, including untranslated sequences, inferred from the full length of the two ESTs is 4,460 basepairs.

## EXAMPLE 7

Preparation of membrane fractions

P2 membrane fractions were prepared at 4°C as follows. Tissues or cells were washed twice with cold PBS, collected by  
5 centrifugation at 100xg for 7 min, and resuspended in 10 ml of buffer A: 5 mM Tris-HCl, 2 mM EDTA containing (1X) protease inhibitor cocktail Complete® tablets (Boehringer Mannheim), pH 7.4 at 4°C. Tissues or cells were disrupted by polytron homogenization, centrifuged at 100xg for 7 min to pellet unbroken cells and nuclei, and the supernatant collected.  
10 The resulting pellet was homogenized a second time in 10 ml of buffer A, centrifuged as described above and supernatant fractions saved. The pooled S1 supernatant was centrifuged at high speed (27 000xg for 20 min) and the pellet was washed once with buffer A, centrifuged (27 000xg for 20 min) and resuspended in buffer A to make the P2 membrane  
15 fraction, and stored at -80°C. Protein content was determined using the Bio-Rad Protein Assay Kit according to manufacturer instructions.

## EXAMPLE 8

Receptor filter-binding assays

20 Competition of [<sup>125</sup>I]CGP71872 binding experiments were performed with ~7 µg P2 membrane protein and increasing concentrations of cold ligand (10<sup>-12</sup>-10<sup>-3</sup> M). The concentration of radioligand used in the competition assays was 1 nM (final). Each concentration was examined in duplicate and incubated for 2 hours at 22°C in the dark in a  
25 total volume of 250 µL binding buffer: 50 mM Tris-HCl, 2.5 mM CaCl<sub>2</sub> (pH 7.4) with (1X) protease inhibitor cocktail Complete® tablets. Bound ligand was isolated by rapid filtration through a Brandel 96 well cell harvester using Whatman GF/B filters. Data were analysed by nonlinear least-squares regression using the computer-fitting program GraphPad  
30 Prism version 2.01 (San Diego).

## EXAMPLE 9

Photoaffinity labelling

P2 membranes were resuspended in binding buffer and  
5 incubated in the dark with 1 nM final concentration [<sup>125</sup>I]CGP71872  
(2200 Ci/mmol) in a final volume of 1 ml for 2 h at 22°C. The membranes  
were centrifuged at 27,000xg for 10 min and the pellet was washed in ice-  
cold binding buffer, centrifuged at 27,000xg for 20 min, resuspended in 1  
ml of ice-cold binding buffer, and exposed on ice 2 inches from 360 nm  
10 ultraviolet light for 10 min. Photolabelled membranes were washed,  
pelleted by centrifugation, and solubilized in sample buffer (50 mM Tris-  
HCl pH 6.5, 10% SDS, 10% glycerol, and 0.003% bromophenol blue with  
10% 2-mercaptoethanol). Samples were electrophoresed on precast  
NOVEX 10% Tris-glycine gels, fixed, dried, and exposed to Kodak XAR  
15 film with an intensifying screen at -70°C.

## EXAMPLE 10

Immunoprecipitation and immunoblotting of GABA<sub>B</sub> receptors

Digitonin solubilized FLAG-tagged HG20 receptors were  
20 immunoprecipitated with a mouse anti-FLAG M2 antibody affinity resin  
(Kodak IBI) and immunoblot analysis conducted as previously described  
(Ng et al., 1996, Biochem. Biophys. Res. Comm. 227:200-204). Following  
washing of the immunoprecipitate, the pellet was resuspended in SDS  
sample buffer and subjected to SDS-PAGE and immunoblotted with  
25 affinity purified GABA<sub>B</sub>R1a-specific antibodies 1713.1 (raised against the  
peptide acetyl-DVNSRRDILPDYELKLC-amide (a portion of  
SEQ.ID.NO.:20)) and 1713.2 (raised against the peptide acetyl-  
CATLHNPTRVKLF EK-amide (a portion of SEQ.ID.NO.:20)).

## EXAMPLE 11

Melanophore functional assay

Growth of *Xenopus laevis* melanophores and fibroblasts was performed as described previously (Potenza et al., 1992, Anal. Biochem. 206:315-322). The cells (obtained from Dr. M.R. Lerner, Yale University) were collected by centrifugation at 200xg for 5 min at 4°C, and resuspended at  $5 \times 10^6$  cells per ml in ice cold 70% PBS, pH 7.0. DNA encoding the relevant GPCR was transiently transfected into melanophores by electroporation using a BTX ECM600 electroporator (Genetronics, Inc., San Diego, CA). To monitor the efficiency of transfection, two internal control GPCRs were used independently (pcDNA1amp-cannabinoid 2 and pcDNA3-thromboxane A2; (Lerner, 1994, Trends Neurosci. 17:142-146)). Cells were electroporated using the following settings: capacitance of 325 microfarad, voltage of 450 volts, and resistance of 720 ohms. Following electroporation, cells were mixed with fibroblast-conditioned growth medium and plated onto flat bottom 96 well microtiter plates (NUNC). 24 hrs after the transfection, the media was replaced with fresh fibroblast-conditioned growth media and incubated for an additional day at 27°C prior to assaying for receptor expression. For Gs/Gq-coupling responses (resulting in pigment dispersion), cells were incubated in 100 µl of 70% L-15 media containing 15 mM HEPES, pH 7.3, and melatonin (0.8 nM final concentration) for 1 hr in the dark at room temperature, and then incubated in the presence of melatonin (0.8 nM final concentration) for 1 h in the dark at room temperature to induce pigment aggregation. For Gi-coupled responses (resulting in pigment aggregation), cells were incubated in the presence of 100 µl/well of 70% L-15 media containing 2.5% fibroblast-conditioned growth medium, 2 mM glutamine, 100 µg/ml streptomycin, 100 units/ml penicillin and 15 mM HEPES, pH 7.3, for 30 min in the dark at room temperature to induce pigment dispersion. Absorbance readings at 600 nm were measured using a Bio-Tek Elx800 Microplate reader (ESBE Scientific) before (Ai) and after

(Af) incubation with ligand (GABA; 1.5 hr in the dark at room temperature).

## EXAMPLE 12

### 5    Stable and transient transfections and determination of cAMP response in HEK293 cells

- HG20 and murine GABA<sub>B</sub>R1a cDNAs were subcloned into pcDNA3.1 (Invitrogen, San Diego, CA) and used to transfect HEK293 cells. Stably expressing cells were identified after selection in geneticin (0.375 mg/ml) by dot blot analysis. For co-expression experiments, the stable cell lines hgb2-42 (expressing HG20) and rgl1a-50 (expressing murine GABA<sub>B</sub>R1a) were transiently transfected with murine GABA<sub>B</sub>R1a and HG20, respectively, in pcDNA3.1 and cells were assayed for cAMP responses.
- 15        Wild-type HEK293 cells, or HEK293 cells stably and transiently expressing HG20 and murine GABA<sub>B</sub>R1a receptors were lifted in 1X PBS, 2.5 mM EDTA, counted, pelleted and resuspended at  $1.5 \times 10^5$  cells per 100  $\mu$ l in Krebs-Ringer-Hepes medium (Blakely et al., 1991, Anal. Biochem. 194:302-308), 100 mM Ro 20-1724 (RBI) and incubated at 20    37°C for 20 min. 100  $\mu$ l of cells was added to 100  $\mu$ l of prewarmed (37°C, 10 min) Krebs-Ringer-Hepes medium, 100 mM Ro 20-1724 without or with agonist and/or 10  $\mu$ M forskolin. Incubations with GABA included 100  $\mu$ M aminooxyacetic acid (a GABA transaminase inhibitor) to prevent breakdown of GABA and 100  $\mu$ M nipecotic acid to block GABA uptake.
- 25    Following a 20 min incubation at 37°C, the assay was terminated by setting the cells on ice and centrifuging at 2,000 rpm for 5 min at 4°C. 175 ml of assay solution was removed and replaced with 175 ml of 0.1 N hydrochloric acid, 0.1 mM calcium chloride and cells were set on ice for 30 min and then stored at -20°C. cAMP determinations were made using a 30    solid phase modification (Maidment et al., 1989, Neurosci. 33:549-557) of the cAMP radioimmunoassay described by Brooker et al. 1979, Adv. Cyclic Nucl. Res. 10:1-33) and previously reported in Clark et al., 1998, Mol.



Endocrinol. 12:193-206). Immulon II removable wells (Dynatech; Chantilly, VA) were coated overnight with 100 µl of protein G (1mg/ml in 0.1M NaHCO<sub>3</sub>, pH 9.0) at 4°C. Prior to use, protein G-coated plates were rinsed with PBS-gelatin-Tween (phosphate buffered saline containing 0.1% gelatin, 0.2% Tween-20) 3 times quickly, and then once for 30 minutes. Following the rinse with PBS-gelatin-Tween, the RIA was set up by adding 100 µl 50 mM sodium acetate, pH 4.75, cAMP standards or aliquots from treated cells, 5,000-7,000 cpm <sup>125</sup>I-succinyl cAMP, and 25 µl of a sheep antibody to cAMP diluted in 50 mM sodium acetate, pH 4.75 (Atto instruments; dilution of stock to 2.5x10<sup>-5</sup>, determined empirically) to the plates in a final volume of 175 µl. Plates were incubated 2 hr at 37°C or overnight at 4°C, rinsed 3 times with sodium acetate buffer, blotted dry, and then individual wells were broken off and bound radioactivity was determined in a gamma counter.

15

### EXAMPLE 13

#### In situ hybridization for co-localization experiments

Preparation of rat brain sections, prehybridization and hybridization of rat brain slices was performed as described previously (Bradley et al., 1992, J. Neurosci. 12:2288-2302; <http://intramural.nimh.nih.gov/lcmr/snge/Protocol.html>). Adjacent coronal rat brain sections were hybridized with labeled antisense and sense riboprobes directed against HG20 (GenBank accession number AF058795) or murine GABABR1a.

HG20 probes were generated by amplification of HG20 with JC216 (T3 promotor/primer and bases 1172-1191) paired with JC217 (T7 promotor/primer and bases 1609-1626) or with JC218 (T3 promotor/primer and bases 2386-2405) paired with JC219 (T7 promotor/primer and bases 2776-2793):  
(JC216: cgcgcaattaaccctcactaaaggACAACAGCAAACGTTTCAGGC (SEQ.ID.NO.:28);

30

JC217: gcgcgtaatacg actcactatagggCATGCCTATGATGGTGAG  
(SEQ.ID.NO.:29);

JC218: cgcgcaattaaccctcactaaagg CTGAGGACAAACCCTGACGC  
(SEQ.ID.NO.:30);

5 JC219: gcgcgtaatacgactcactatagggGATGTC TTCTATGGGGTC;  
(SEQ.ID.NO.:31)).

Murine GABA $\beta$ R1a probes were generated by amplification  
of murine GABA $\beta$ R1a with JC160 (T3 promotor/primer and bases 631-  
648) paired with JC161 (T7 promotor/primer and bases 1024-1041):

10 (JC160: cgcgcaattaaccctcactaaaggAAGCTTATCCACCACGAC  
(SEQ.ID.NO.:32);

JC161:gcgcgtaa tacgactcactatagggAGCTGGATCCGAGAAGAA  
(SEQ.ID.NO.:33)).

For colocalization experiments, murine GABA $\beta$ R1a probes  
15 were labeled with digoxigenin-UTP and detected using a peroxidase-  
conjugated antibody to digoxigenin and TSA amplification involving  
biotinyl tyramide and subsequent detection with streptavidin-conjugated  
fluorescein. HG20 probes were radiolabelled  
([http://intramural.nimh.nih.gov/lcmr/snge/Protocol](http://intramural.nimh.nih.gov/lcmr/snge/Protocol.html).  
20 [html](http://intramural.nimh.nih.gov/lcmr/snge/Protocol.html)). For individual hybridizations, murine GABA $\beta$ R1a and HG20  
riboprobes were radiolabeled with <sup>35</sup>S-UTP and detected as described  
previously (Bradley et al., 1992, J. Neurosci. 12:2288-2302;  
<http://intramural.nimh.nih.gov/lcmr/snge/Protocol.html>). Brain slices  
were either hybridized with individual radiolabelled probes or, for  
25 colocalization studies, simultaneously with probes to both murine  
GABA $\beta$ R1a and HG20 receptors. Detection of the radiolabeled HG20  
probe was performed after detection of the digoxigenin-labeled rgb1 probe  
on the same brain slices.

## EXAMPLE 14

Construction of N-terminal and C-terminal fragments of murine GABA<sub>B</sub>R1a

The N-terminal fragment of murine GABA<sub>B</sub>R1a, comprising  
5 amino acid positions 1-625, was generated by PCR. The coding sequence of  
the N-terminal fragment was amplified by using primer pairs: NFP-  
CJ7843F139 (5'- ACC ACT GCT AGC ACC GCC ATG CTG CTG CTG  
CTG CTT CTG C -3'; SEQ.IS.NO.:34) and NRP-CJ7844 (3'- GG GTG CGA  
GCA ATA TAG GTC TTA AGG GTC GGC CGC CGG CGT CAC CA -5'; ;  
10 SEQ.IS.NO.:35). Similarly, the C-terminal fragment, amino acid positions  
588-942, was generated by PCR using primer pairs: CFP-CJ7845 (5'- ACC  
ACT GCT AGC ACC GCC ATG CAG AAA CTC TTT ATC TCC GTC TCA  
GTT CTC TCC AGC-3'; ; SEQ.IS.NO.:36) and CRP-CJ7846 (3'- CAG CTC  
ATG TAA ACG AAA TGT TCA CTC GCC GGC CGC CGG CGT CAC CA-  
15 5'; ; SEQ.IS.NO.:37). PCR reactions were carried out using the  
Advantage-HF PCR kit (Clontech, Paolo Alto, CA) with 0.2 ng of murine  
GABA<sub>B</sub>R1a DNA as the template, and 10 µM of each primer according to  
manufacturer instructions. The PCR conditions were as follows: precycle  
denaturation at 94°C for 1 min, and then 35 cycles at 94°C (15 s),  
20 annealing and extension at 72°C (3 min), followed by a final extension for  
3 min at 72°C. The PCR products, N-gb 1a and C-gb 1a DNA, flanked by  
Nhe1 and Not1 sites, were digested and subcloned into the Nhe1/Not1 site  
of pcDNA3.1 (Invitrogen, San Diego, Ca).

25

## EXAMPLE 15

Cell culture and preparation of membrane fractions for binding experiments using N-terminal and C-terminal GABA<sub>B</sub>R1a fragments

COS-7 cells (ATCC) were cultured in DMEM, 10% bovine  
serum, 25 mM HEPES, and antibiotics and transiently transfected with  
30 murine gb1a/pcDNA3.1 (encoding full-length GABA<sub>B</sub>R1a), N-gb

1a/pcDNA3.1 (encoding the N-terminal fragment of GABA<sub>B</sub>R1a; see Example 14) or C-gb 1a/pcDNA3.1 (encoding the C-terminal fragment of GABA<sub>B</sub>R1a; see Example 14) using Lipofectamine reagent (Gibco BRL) following the conditions recommended by the manufacturer. At 48 h post-transfection, P2 membrane fractions were prepared at 4°C as follows: Cells were washed twice with cold PBS, collected by centrifugation at 100xg for 7 min, and resuspended in 10 ml of buffer A: 5 mM Tris-HCl, 2 mM EDTA containing (1X) protease inhibitor cocktail Complete<sup>®</sup> tablets (Boehringer Mannheim), pH 7.4 at 4°C. Cells were disrupted by polytron homogenization, centrifuged at 100xg for 7 min to pellet unbroken cells and nuclei, and the supernatant collected. The resulting pellet was homogenized a second time in 10 ml of buffer A, centrifuged as described above and supernatant fractions saved. The pooled S1 supernatant was centrifuged at high speed (27,000xg for 20 min) and the pellet was washed once with buffer A, centrifuged (27,000xg for 20 min), resuspended in buffer A to make the P2 membrane fraction, and stored at -80°C. Protein content was determined using the Bio-Rad Protein Assay Kit according to manufacturer instructions.

20

## EXAMPLE 16

*In vitro* transcription/translation of GABA<sub>B</sub>R1a and N-terminal and C-terminal fragments

*In vitro* transcription coupled translation reactions were performed in the presence of [<sup>35</sup>S]-methionine in the TNT Coupled Reticulocyte Lysate system (Promega, WI) using the pcDNA3.1 plasmid containing the full-length GABA<sub>B</sub>R1a, N-gb1a, and C-gb1a DNAs. Translation products were analysed by electrophoresis on 8-16% Tris-Glycine gradient gels (Novex pre-cast gel system) under denaturing and reducing conditions. Gels were fixed, treated with enlightening fluid (NEN), dried and exposed to Kodak X-AR film at -70°C for 4 to 24 h. Analysis of the results of these *in vitro* transcription coupled translation reactions confirmed that the constructs whose production is described in Example 14 directed the

expression of the appropriate GABA<sub>B</sub>R1a fragments (see Figure 17A).

### EXAMPLE 17

5 Immunoblot analysis for experiments with N-terminal and C-terminal fragments of GABA<sub>B</sub>R1a

The expression of full-length and N-terminal and C-terminal GABA<sub>B</sub>R1a fragments *in vivo* was confirmed by immunoblot analysis. Membranes were solubilized in SDS sample buffer consisting of 50 mM Tris-HCl pH 6.5, 10% SDS, 10% glycerol, and 0.003% bromophenol blue  
10 with 10% 2-mercaptoethanol and separated on SDS-PAGE. The full-length receptor and N-terminal fragment were detected using affinity purified rabbit GABA<sub>B</sub>R1a polyclonal antibody 1713.1 (acetyl-DVNSRRDILPDYELKLC-amide; a portion of SEQ.ID.NO.:20) and 1713.2 (acetyl-CATLHNPTRVKLF EK-amide; a portion of SEQ.ID.NO.:20)  
15 (Quality Control Biochemicals (Hopkinton, MA). The C-terminal fragment was detected using a GABA<sub>B</sub>R1a antibody raised against the C-terminal tail of the receptor (acetyl-PSEPPDRLSCDGSRVHLLYK-amide; SEQ.ID.NO.:20) (Chemicon Int., Inc., Canada).

20

### EXAMPLE 18

Receptor filter-binding assays for experiments with N-terminal and C-terminal fragments of GABA<sub>B</sub>R1a

Competition of [<sup>125</sup>I] CGP71872 binding experiments were performed with ~7 µg P2 membrane protein and increasing  
25 concentrations of cold ligand (10<sup>-12</sup>-10<sup>-3</sup> M). The concentration of radioligand used in the competition assays was 1 nM (final). Each concentration was examined in duplicate and incubated for 2 hr at 22°C in the dark in a total volume of 250 µL binding buffer: 50 mM Tris-HCl, 2.5 mM CaCl<sub>2</sub> (pH 7.4) with (1X) protease inhibitor cocktail Complete®  
30 tablets. Bound ligand was isolated by rapid filtration through a Brandel

96 well cell harvester using Whatman GF/B filters. Data were analysed by nonlinear least-squares regression using the computer-fitting program GraphPad Prism version 2.01 (San Diego).

5

## EXAMPLE 19

Photoaffinity labeling for experiments with N-terminal and C-terminal fragments of GABABR1a

P2 membranes were resuspended in binding buffer, and incubated in the dark with 1 nM final concentration [<sup>125</sup>I]CGP71872 (2200 Ci/mmol) in a final volume of 1 ml for 2 h at 22°C. The membranes were centrifuged at 27,000xg for 10 min and the pellet was washed in ice-cold binding buffer, centrifuged at 27,000xg for 20 min and resuspended in 1 ml of ice-cold binding buffer and exposed on ice 2 inches from 360 nm ultraviolet light for 10 min. Photolabeled membranes were washed and membranes pelleted by centrifugation and solubilized in sample buffer (50 mM Tris-HCl pH 6.5, 10% SDS, 10% glycerol, and 0.003% bromophenol blue with 10% 2-mercaptoethanol). Samples were electrophoresed on precast NOVEX 10% Tris-glycine gels, fixed, dried, and exposed to Kodak XAR film with an intensifying screen at -70°C.

20

## EXAMPLE 20

Construction of the FLAG epitope-tagged HG20 and detection of expression in vitro and in COS-1 cells

25

The FLAG epitope-tagged HG20 receptor subunit was constructed by PCR using a sense primer encoding a modified influenza hemagglutinin signal sequence (MKTIIALSYIFCLVFA; a portion of SEQ.ID.NO.:17) (Jou et al., 1980, Cell 19:683-696) followed by an antigenic FLAG epitope (DYKDDDDK; a portion of SEQ.ID.NO.:17) and DNA encoding amino acids 52-63 of HG20 and an antisense primer encoding amino acids 930-941 of the HG20 in a high-fidelity PCR reaction

30

with HG20/pCR 3.1 as a template. HG20/pCR 3.1 is a plasmid that contains full-length HG20 (SEQ.ID.NO.:2) cloned into pCR3.1. The nucleotide sequences of the sense and antisense primers are: sense: 5'-GCC GCT AGC GCC ACC ATG AAG ACG ATC ATC GCC CTG AGC TAC  
5 ATC TTC TGC CTG GTA TTC GCC GAC TAC AAG GAC GAT GAT GAC  
AAG AGC AGC CCG CCG CTC TCC ATC ATG GGC CTC ATG CCG CTC-  
3', (SEQ.ID.NO.:38); antisense: 5'-GCC TCT AGA TTA CAG GCC CGA  
GAC CAT GAC TCG GAA GGA GGG TGG CAC-3'. (SEQ.ID.NO.:39). The PCR conditions were: precycle denaturation at 94°C for 1 min, 94°C for 30  
10 sec, annealing and extension at 72°C for 4 min for 25 cycles, followed by a 7 min extension at 72°C. The PCR product, SF-HG20 DNA, flanked by NheI and XbaI sites was subcloned into the NheI/XbaI site of pcDNA3.1 (Invitrogen, San Diego, Ca) to give rise to the expression construct SF-HG20/pcDNA3.1. The sequence of this construct was verified on both  
15 strands.

The SF-HG20 receptor was expressed in an *in vitro* coupled transcription/translation reaction using the TNT Coupled Reticulocyte Lysate system (Promega, WI) in the presence of [<sup>35</sup>S]methionine according to the manufacturer instructions. Radiolabeled proteins were  
20 analyzed by electrophoresis on 8-16% Tris-Glycine gradient gels (Novex pre-cast gel system) under denaturing and reducing conditions. Gels were fixed and treated with Enlightening fluid (NEN), dried and exposed to Kodak X-AR film at -70°C.

COS-1 cells (ATCC, CRL 1650) were cultured in DMEM, 10%  
25 bovine serum, 25 mM HEPES, pH 7.4, and 10 units/mL penicillin- 10 µg/mL streptomycin. Transient transfection of COS-1 cells with SF-HG20/pcDNA 3.1 was carried out using Lipofectamine reagent (Gibco BRL) following the conditions recommended by the manufacturer. At 48 h post-transfection, crude membranes were prepared and receptors were  
30 solubilized with digitonin and immunoprecipitated with anti-FLAG M2 affinity gel resin (IBI) under previously described conditions (Ng et al., 1993). The immunoprecipitate was washed and solubilized in SDS sample buffer, sonicated, electrophoresed, and blotted on to nitrocellulose membrane as described (Ng et al., 1993). The FLAG-tagged HG20

receptor was detected using an anti-FLAG antibody (Santa Cruz Biotech., Inc.) by following a chemilumescence protocol of the manufacturer (NEN).

## EXAMPLE 21

### 5 Kir channel activity in *Xenopus* oocytes

With the following modifications, *Xenopus* oocytes were isolated as described (Hébert et al., 1994, Proc. R. Soc. Lond. B 256:253-261) from live frogs supplied by Boreal, Inc. After a brief (10 min) hypertonic shock with 125 mM potassium phosphate pH 6.5, oocytes were  
10 allowed to recover in Barth's solution for 1-2 hr. cDNA constructs for human Kir 3.1, Kir 3.2 channel isoforms (generous gifts from Dr. Hubert Van Tol, University of Toronto), and  $G\alpha 1$  (a generous gift of Dr. Maureen Linder, Washington University) were linearized by restriction enzymes and purified using GeneClean (Bio 101). Murine GABAR1a or FLAG-  
15 HG20 clones were subcloned into pT7TS (a generous gift of Dr. Paul Krieg, University of Texas) before linearization and transcription. Capped cRNA was made using T7 RNA polymerase and the mMessage mMachine (Ambion). Individual oocytes were injected with 5-10 ng (in 25-50 nL) of Kir3.1 and Kir3.2 constructs with mRNAs for murine  
20 GABAR1a or FLAG-HG20 and in combination with  $G\alpha 1$  as well. Kir currents were also evaluated in oocytes co-injected with Kir3.1, Kir3.2, murine GABAR1a and FLAG-HG20 mRNAs. Currents were recorded after 48 hr. Standard recording solution was KD-98, 98 mM KCl, 1 mM  $MgCl_2$ , 5 mM K-HEPES, pH 7.5, unless otherwise stated. Microelectrodes  
25 were filled with 3 M KCl and had resistances of 1-3 MW and 0.1-0.5 MW for voltage and current electrodes, respectively. In addition, current electrodes were backfilled with 1% agarose (in 3M KCl) to prevent leakage as described (Hébert et al., 1994, Proc. R. Soc. Lond. B 256:253-261). Recordings were made at room temperature using a Geneclamp 500  
30 amplifier (Axon Instruments). Oocytes were voltage clamped and perfused continuously with different recording solutions. Currents were evoked by 500 msec voltage commands from a holding potential of -10



mV, delivered in 20 mV increments from -140 to 60 mV to test for inward rectifying potassium currents. Data were recorded at a holding potential of -80 mV and drugs were added to the bath with a fast perfusion system. Data collection and analysis were performed using pCLAMP v6.0 (Axon Instruments) and Origin v4.0 (MicroCal) software. For subtraction of endogenous and leak currents, records were obtained in ND-96, 96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM Na-HEPES and these were subtracted from recordings in KD-98 before further analysis.

10

## EXAMPLE 22

Radiation Hybrid mapping of HG20

Radiation hybrid analysis assigned the HG20 gene to chromosome 9, placing it 4.81 cR from the WI-8684 marker on the GeneBridge 4 panel of 93 RH clones of the whole human genome. Searching of the OMIM database with D9S176 and D9S287 markers proximal to the HG20 gene revealed it to map proximal to the hereditary sensory neuropathy type 1 (HSN-1) locus, a ~8 cM region flanked by D9S176 and 9S318 (Nicholson et al., 1996, Nature Genetics 13, 101-104) (Figure 20). HSN-1 is the most common form of a group of degenerative disorders of sensory neurons characterized by a progressive degeneration of dorsal root ganglion and motor neurons that lead to distal sensory loss, distal muscle wasting and weakness, and neural deafness, among a number of other neuronally related deficits (Nicholson et al., 1996, Nature Genetics 13, 101-104). FCMD (Fukuyama congenital muscular dystrophy) and DYS (dysautonomia, another type of HSN) also map to this area. Candidate gene(s) in these disorders are likely critical to the development, survival, and differentiation of neurons.

A human BAC library was screened using the EcoRI fragment containing the full-length HG20 DNA, and end-sequencing was performed on BAC clones designated 6D18, 168K19, 486B24, and 764N4. The primer pair: ngflt7+ (5'-AAC AGT CAA AAC CCA CCC AG-3'; SEQ.ID.NO.:40) and ngflt7- (5'-AAC AGT TTC CAG CTG TGC CT-3';

SEQ.ID.NO.:41) were identified for radiation hybrid mapping of the HG20 gene on the GENEBRIDGE 4 panel. BAC library screening and radiation hybrid mapping were performed by Research Genetics (Huntsville, AL).

- 5                   The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.
- 10                   Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

## WHAT IS CLAIMED:

1. An isolated DNA molecule encoding an HG20 polypeptide comprising an amino acid sequence selected from the group consisting of:

5  
10  
15  
SEQ.ID.NO.:2;  
Positions 9-941 of SEQ.ID.NO.:2;  
Positions 35-941 of SEQ.ID.NO.:2;  
Positions 36-941 of SEQ.ID.NO.:2;  
Positions 38-941 of SEQ.ID.NO.:2;  
Positions 39-941 of SEQ.ID.NO.:2;  
Positions 42-941 of SEQ.ID.NO.:2;  
Positions 44-941 of SEQ.ID.NO.:2;  
Positions 46-941 of SEQ.ID.NO.:2;  
Positions 52-941 of SEQ.ID.NO.:2; and  
Positions 57-941 of SEQ.ID.NO.:2.

2. The isolated DNA molecule of claim 1 comprising a nucleotide sequence selected from the group consisting of:

20  
25  
30  
SEQ.ID.NO.:1;  
Positions 293-3,115 of SEQ.ID.NO.:1;  
Positions 317-3,115 of SEQ.ID.NO.:1;  
Positions 395-3,115 of SEQ.ID.NO.:1;  
Positions 398-3,115 of SEQ.ID.NO.:1;  
Positions 404-3,115 of SEQ.ID.NO.:1;  
Positions 407-3,115 of SEQ.ID.NO.:1;  
Positions 416-3,115 of SEQ.ID.NO.:1;  
Positions 422-3,115 of SEQ.ID.NO.:1;  
Positions 428-3,115 of SEQ.ID.NO.:1;  
Positions 446-3,115 of SEQ.ID.NO.:1; and  
Positions 461-3,115 of SEQ.ID.NO.:1.

3. An isolated DNA molecule that hybridizes under stringent conditions to the DNA molecule of claim 2.

4. An expression vector comprising the DNA of claim 1.
5. A recombinant host cell comprising the expression vector of claim 4.
6. The recombinant cell of claim 5 further comprising an expression vector comprising DNA encoding a protein selected from the group consisting of:
7. A protein, substantially free from other proteins, comprising an HG20 protein having an amino acid sequence selected from the group consisting of:
- SEQ.ID.NO.:2;  
Positions 9-941 of SEQ.ID.NO.:2;  
Positions 35-941 of SEQ.ID.NO.:2;  
Positions 36-941 of SEQ.ID.NO.:2;  
Positions 38-941 of SEQ.ID.NO.:2;  
Positions 39-941 of SEQ.ID.NO.:2;  
Positions 42-941 of SEQ.ID.NO.:2;  
Positions 44-941 of SEQ.ID.NO.:2;  
Positions 46-941 of SEQ.ID.NO.:2;  
Positions 52-941 of SEQ.ID.NO.:2; and  
Positions 57-941 of SEQ.ID.NO.:2.
8. A heterodimer comprising the protein of claim 7 and a G-protein coupled receptor protein where the heterodimer is substantially free from other proteins.
9. The heterodimer of claim 8 where the heterodimer is held together by N-terminal Sushi repeats, C-terminal alpha-helical

interacting domains, coiled-coil domains, transmembrane interactions, or disulfide bonds.

10. A polypeptide comprising a coiled-coil domain from a first GABA<sub>B</sub> receptor subunit and no other contiguous amino acid sequences longer than 5 amino acids from the first GABA<sub>B</sub> receptor subunit where the coiled-coil domain is present in the C-terminus of the GABA<sub>B</sub> receptor subunit and mediates heterodimerization of the first GABA<sub>B</sub> receptor subunit with a second GABA<sub>B</sub> receptor subunit.

10

11. The polypeptide of claim 10 where the coiled-coil domain is selected from the group consisting of: positions 756-829 of SEQ.ID.NO.:2; positions 779-814 of SEQ.ID.NO.:2; positions 886-949 of SEQ.ID.NO.:21; and positions 889-934 of SEQ.ID.NO.:21.

15

12. An isolated DNA molecule encoding a GABA<sub>B</sub>R1a polypeptide comprising the amino acid sequence SEQ.ID.NO.:20.

13. A protein, substantially free from other proteins, comprising a GABA<sub>B</sub>R1a protein having the amino acid sequence SEQ.ID.NO.:20.

20

14. A method for determining whether a substance binds GABA<sub>B</sub> receptors and is thus a potential agonist or antagonist of the GABA<sub>B</sub> receptor that comprises:

25

(a) providing cells comprising an expression vector encoding HG20 and an expression vector encoding GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b;

(b) culturing the cells under conditions such that HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b are expressed and heterodimers of HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b are formed;

30

(c) exposing the cells to a labeled ligand of GABA<sub>B</sub> receptors in the presence and in the absence of the substance;

(d) measuring the binding of the labeled ligand to the heterodimers of HG20 and GABABR1a or GABABR1b;

where if the amount of binding of the labeled ligand is less in the presence of the substance than in the absence of the substance, then  
5 the substance is a potential agonist or antagonist of GABAB receptors.

15. A method of identifying agonists and antagonists of HG20 comprising:

- (a) providing test cells by transfecting cells with:  
10 (1) an expression vector that directs the expression of HG20 in the cells; and  
(2) an expression vector that directs the expression of GABABR1a or GABABR1b in the cells;  
(b) exposing the test cells to a substance that is suspected  
15 of being an agonist of the GABAB receptor;  
(c) measuring the amount of a functional response of the test cells that have been exposed to the substance;  
(d) comparing the amount of the functional response exhibited by the test cells with the amount of the functional response  
20 exhibited by control cells;

wherein if the amount of the functional response exhibited by the test cells differs from the amount of the functional response exhibited by the control cells, the substance is an agonist or antagonist of the GABAB receptor;

25 where the control cells are cells that have not been transfected with HG20 and GABABR1a or GABABR1b but have been exposed to the substance or are test cells that have not been exposed to the substance.

30 16. A method of producing functional GABAB receptors in cells comprising:

- (a) transfecting cells with:  
(1) an expression vector that directs the expression of HG20 in the cells; and

(2) an expression vector that directs the expression of GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b in the cells;

(b) culturing the cells under conditions such that heterodimers of HG20 and GABA<sub>B</sub>R1a or GABA<sub>B</sub>R1b are formed where the heterodimers constitute functional GABA<sub>B</sub> receptors.

17. An antibody that binds specifically to HG20 where HG20 has an amino acid sequence selected from the group consisting of:

SEQ.ID.NO.:2;

Positions 9-941 of SEQ.ID.NO.:2;

Positions 35-941 of SEQ.ID.NO.:2;

Positions 36-941 of SEQ.ID.NO.:2;

Positions 38-941 of SEQ.ID.NO.:2;

Positions 39-941 of SEQ.ID.NO.:2;

Positions 42-941 of SEQ.ID.NO.:2;

Positions 44-941 of SEQ.ID.NO.:2;

Positions 46-941 of SEQ.ID.NO.:2;

Positions 52-941 of SEQ.ID.NO.:2; and

Positions 57-941 of SEQ.ID.NO.:2.

18. A method of expressing a truncated version of HG20 protein comprising:

(a) transfecting a host cell with an expression vector that encodes an HG20 protein that has been truncated at the amino terminus;

(b) culturing the transfected cells of step (a) under conditions such that the truncated HG20 protein is expressed.

19. A chimeric HG20 protein having an amino acid sequence of HG20 selected from the group consisting of:

Positions 51-941 of SEQ.ID.NO.:2;

Positions 52-941 of SEQ.ID.NO.:2;

Positions 53-941 of SEQ.ID.NO.:2;

Positions 54-941 of SEQ.ID.NO.:2;

Positions 55-941 of SEQ.ID.NO.:2;

Positions 56-941 of SEQ.ID.NO.:2;  
Positions 57-941 of SEQ.ID.NO.:2; and  
Positions 58-941 of SEQ.ID.NO.:2;  
covalently linked at the N-terminus with a non-HG20 amino acid  
5 sequence.



1/35

1 CCGCCCTCCC CCGGCCGAGC TCCAGGGCTG CCGCCTAGCA GCTCCCGGCC  
51 GGAGAGCGGT TCAGAGCTCG CTCCACCCC TTCCCGGCGT GATTGATCCG  
101 TCACGGGCGC CTCCGCTGCC GCCGCCGCCG CCGCGGCCGT TCTGAGCCGA  
151 GCCGGAACCC TAGCCCGAGA CGGAGCCGGG GCCCGGGCCG GCGCCATTGC  
201 GCGGGCGCCG CGGGAAGACC TTGGCGCGGG GCGGCGGGCC GGGCCAGGCC  
251 ATGCGGGCCG AGTGAGCCGG CGCCCGCAGC CCGCGGCGCG GCATGGCTTC  
301 CCCGCGGAGC TCCGGGCAGC CCGGGCCGCC GCCGCCGCCG CCACCGCCGC  
351 CCGCGCGCCT GCTACTGCTA CTGCTGCTGC CGCTGCTGCT GCCTCTGGCG  
401 CCCGGGGCCT GGGGCTGGGC GCGGGGCGCC CCCC GGCCGC CGCCCAGCAG  
451 CCCGCCGCTC TCCATCATGG GCCTCATGCC GCTCACCAAG GAGGTGGCCA  
501 AGGGCAGCAT CGGGCGCGGT GTGCTCCCCG CCGTGGAAGT GGCCATCGAG  
551 CAGATCCGCA ACGAGTCACT CCTGCGCCCC TACTTCCTCG ACCTGCGGCT  
601 CTATGACACG GAGTGCGACA ACGCAAAAGG GTTGAAAGCC TTCTACGATG  
651 CAATAAAATA CGGGCCGAAC CACTTGATGG TGTTTGGAGG CGTCTGTCCA  
701 TCCGTCACAT CCATCATTGC AGAGTCCCTC CAAGGCTGGA ATCTGGTGCA  
751 GCTTTCTTTT GCTGCAACCA CGCCTGTTCT AGCCGATAAG AAAAAATACC  
801 CTTATTTCTT TCGGACCGTC CCATCAGACA ATGCGGTGAA TCCAGCCATT  
851 CTGAAGTTGC TCAAGCACTA CCAGTGGAAG CGCGTGGGCA CGCTGACGCA  
901 AGACGTTTCA AGGTTCTCTG AGGTGCGGAA TGACCTGACT GGAGTTCTGT  
951 ATGGCGAGGA CATTGAGATT TCAGACACCG AGAGCTTCTC CAACGATCCC  
1001 TGTACCAAGT TCAAAAAGCT GAAGGGGAAT GATGTGCGGA TCATCCTTGG  
1051 CCAGTTTGAC CAGAATATGG CAGCAAAAGT GTTCTGTTGT GCATACGAGG  
1101 AGAACATGTA TGGTAGTAAA TATCAGTGGA TCATTCCGGG CTGGTACGAG  
1151 CCTTCTTGGT GGGAGCAGGT GCACACGGAA GCCAACTCAT CCCGCTGCCT  
1201 CCGGAAGAAT CTGCTTGCTG CCATGGAGGG CTACATTGGC GTGGATTTCTG  
1251 AGCCCCTGAG CTCCAAGCAG ATCAAGACCA TCTCAGGAAA GACTCCACAG  
1301 CAGTATGAGA GAGAGTACAA CAACAAGCGG TCAGGCGTGG GGCCCAGCAA  
1351 GTTCCACGGG TACGCCTACG ATGGCATCTG GGTCATCGCC AAGACACTGC  
1401 AGAGGGCCAT GGAGACACTG CATGCCAGCA GCCGGCACCA GCGGATCCAG  
1451 GACTTCAACT ACACGGACCA CACGCTGGGC AGGATCATCC TCAATGCCAT  
1501 GAACGAGACC AACTTCTTCG GGGTCACGGG TCAAGTTGTA TTCCGGAATG  
1551 GGGAGAGAAT GGGGACCATT AAATTTACTC AATTTCAAGA CAGCAGGGAG  
1601 GTGAAGGTGG GAGAGTACAA CGCTGTGGCC GACACACTGG AGATCATCAA  
1651 TGACACCATC AGGTTCCAAG GATCCGAACC ACCAAAAGAC AAGACCATCA  
1701 TCCTGGAGCA GCTGCGGAAG ATCTCCCTAC CTCTCTACAG CATCCTCTCT  
1751 GCCCTCACCA TCCTCGGGAT GATCATGGCC AGTGCTTTTC TCTTCTTCAA  
1801 CATCAAGAAC CGGAATCAGA AGCTCATAAA GATGTGAGT CCATACATGA  
1851 ACAACCTTAT CATCCTTGGA GGGATGCTCT CCTATGCTTC CATATTTCTC  
1901 TTTGGCCTTG ATGGATCCTT TGTCTTGAA AAGACCTTTG AAACACTTTG  
1951 CACCGTCAGG ACCTGGATTG TCACCGTGGG CTACACGACC GCTTTTGGGG  
2001 CCATGTTTGC AAAGACCTGG AGAGTCCACG CCATCTTCAA AAATGTGAAA  
2051 ATGAAGAAGA AGATCATCAA GGACCAGAAA CTGCTTGTGA TCGTGGGGGG  
2101 CATGCTGCTG ATCGACCTGT GTATCCTGAT CTGCTGGCAG GCTGTGGACC  
2151 CCCTGCGAAG GACAGTGGAG AAGTACAGCA TGGAGCCGGA CCCAGCAGGA

FIG. 1A

SUBSTITUTE SHEET (RULE 26)

2/35

2201 CGGGATATCT CCATCCGCCC TCTCCTGGAG CACTGTGAGA ACACCCATAT  
2251 GACCATCTGG CTTGGCATCG TCTATGCCTA CAAGGGACTT CTCATGTTGT  
2301 TCGGTTGTTT CTTAGCTTGG GAGACCCGCA ACGTCAGCAT CCCC GCACTC  
2351 AACGACAGCA AGTACATCGG GATGAGTGTC TACAACGTGG GGATCATGTG  
2401 CATCATCGGG GCCGCTGTCT CCTTCCTGAC CCGGGACCAG CCCAATGTGC  
2451 AGTTCTGCAT CGTGGCTCTG GTCATCATCT TCTGCAGCAC CATCACCTC  
2501 TGCCTGGTAT TCGTGCCGAA GCTCATCACC CTGAGAACAA ACCCAGATGC  
2551 AGCAACGCAG AACAGGCGAT TCCAGTTCAC TCAGAATCAG AAGAAAGAAG  
2601 ATTCTAAAAC GTCCACCTCG GTCACCAGTG TGAACCAAGC CAGCACATCC  
2651 CGCCTGGAGG GCCTACAGTC AGAAAACCAT CGCCTGCGAA TGAAGATCAC  
2701 AGAGCTGGAT AAAGACTTGG AAGAGGTCAC CATGCAGCTG CAGGACACAC  
2751 CAGAAAAGAC CACCTACATT AAACAGAACC ACTACCAAGA GCTCAATGAC  
2801 ATCCTCAACC TGGGAACTT CACTGAGAGC ACAGATGGAG GAAAGGCCAT  
2851 TTTAAAAAAT CACCTCGATC AAAATCCCCA GCTACAGTGG AACACAACAG  
2901 AGCCCTCTCG AACATGCAAA GATCCTATAG AAGATATAAA CTCTCCAGAA  
2951 CACATCCAGC GTCGGCTGTC CCTCCAGCTC CCCATCCTCC ACCACGCCTA  
3001 CCTCCCATCC ATCGGAGGCG TGGACGCCAG CTGTGTCAGC CCCTGCGTCA  
3051 GCCCCACCGC CAGCCCCCGC CACAGACATG TGCCACCCTC CTTCCGAGTC  
3101 ATGGTCTCGG GCCTGTAAGG GTGGGGGGCC TGGGCCCGGG GCCTCCCCCG  
3151 TGACAGAACC AACTGGGCA GAGGGGTCTG CTGCAGAAAC ACTGTGCGGT  
3201 CTGGCTGCGG AGAAGCTGGG CACCATGGCT GGCCTCTCAG GACCACTCGG  
3251 ATGGCACTCA GGTGGACAGG ACGGGGCAGG GGGAGAGTTG GCACCTGACC  
3301 TCGAGCCTTA TTTGTGAAGT CCTTATTTCT TCACAAAGAA GAGGAACGGA  
3351 AATGGGACGT CTTCTTAAC ATCTGCAAAC AAGGAGGCGC TGGGATATCR  
3401 AATTCCACCA CACTGGCGGC CCGCGCTTGS TCSTAATCAT GGTCACTAAT  
3451 GTTTCCTGTG TTGAAATTGT TATCCGCTCC

FIG. 1B

SUBSTITUTE SHEET (RULE 26)

1 MASPRSSGQP GPPPPPPPPP ARLLLLLLLP LLLPLAPGAW GWARGAPRPP  
51 PSSPPLSIMG LMPLTKEVAK GSIGRGVLP VELAIEQIRN ESLLRPYFLD  
101 LRLYDTECDN AKGLKAFYDA IKYGNHLMV FGGVCPSVTS IIAESLQGWN  
151 LVQLSFAATT PVLADKKKYP YFFRTVPSDN AVNPAILKLL KHYQWKRVTG  
201 LTQDVQRFSE VRNDLTGVLY GEDIEISDTE SFSNDPCTSV KKLKGN DVRI  
251 ILGQFDQNMA AKVFCCAYEE NMYGSKYQWI IPGWYEPSWW EQVHTEANSS  
301 RCLRKNLLAA MEGYIGVDFE PLSSKQIKTI SGKTPQQYER EYNNKRSGVG  
351 PSKFHGYAYD GIWVIAKTQ RAMETLHASS RHQRIQDFNY TDHTLGRIL  
401 NAMNETNFFG VTGQVFRNG ERMGTIKFTQ FQDSREVKVG EYNAVADTLE  
451 IINDTIRFQG SEPPKDKTII LEQLRKISLP LYSILSALT I LGMIMASAF  
501 FFNKNRNQK LIKMSSPYMN NLIILGGMLS YASIFLFGD GSFVSEKTFE  
551 TLCTVRTWIL TVGYTTAFGA MFAKTWRVHA IFKNVKMKKK I IKDQKLLVI  
601 VGGMLLIDLC ILICWQAVDP LRRTVEKYSM EPDPAGRDIS IRPLLEHCEN  
651 THMTIWLIV YAYKGLMLF GCFLAWETRN VSIPALNSK YIGMSVYNG  
701 IMCIIGAAS FLTRDQPNVQ FCIVALVIIF CSTITLCLVF VPKLITLRTN  
751 PDAATQNRFF QFTQNQKKED SKTSTSVTSV NQASTSRLEG LOSENHRLRM  
801 KITELDKLE EVTMQLQDTP EKT TYIKQNH YQELNDILNL GNFTSTG  
851 KAILKNHLDQ NPQLQWNTTE PSRTCKDPIE DINSPEHIQR RLSLQLPILH  
901 HAYLPSIGGV DASCVSPCVS PTASPRHRHV PPSFRVMVSG L

FIG. 2

SUBSTITUTE SHEET (RULE 26)

4/35

Sequence: LPLLLPLAPGAWG-WARGAPRPPPSPPLSIMGLMPLTKEVAKGSIGRGVLPVELAIEQIRNE  
|((signal))|(mature peptide)  
29 42

Other entries above 3.50

Score 11.1 at residue 39

Sequence: LLLLLPLLLPLAPG-AWGWARGAPRPPPSPPLSIMGLMPLTKEVAKGSIGRGVLPVELAIEQI  
|((signal))|(mature peptide)  
26 39

Score 8.6 at residue 38

Sequence: LLLLLPLLLPLAP-GAWGWARGAPRPPPSPPLSIMGLMPLTKEVAKGSIGRGVLPVELAIEQ  
|((signal))|(mature peptide)  
25 38

Score 8.1 at residue 35

Sequence: RLILLLLPLLLP-LAPGAWGWARGAPRPPPSPPLSIMGLMPLTKEVAKGSIGRGVLPVELA  
|((signal))|(mature peptide)  
22 35

Score 7.9 at residue 36

Sequence: LILLLLPLLLPL-APGAWGWARGAPRPPPSPPLSIMGLMPLTKEVAKGSIGRGVLPVELAI  
|((signal))|(mature peptide)  
23 36

Score 6.2 at residue 9

Sequence: -QPGRPPPPPPPPARLILLLLPLLLPLAPGAWGWARGAPRPPPSPPLSI  
|((signal))|(mature peptide)  
-4 9

Score 5.7 at residue 46

Sequence: LPLAPGAWGWARG-APRPPPSPPLSIMGLMPLTKEVAKGSIGRGVLPVELAIEQIRNESLLR  
|((signal))|(mature peptide)  
33 46

Score 5.6 at residue 747

Sequence: ITLCLVFVPKLIT-LRTNPDAATQNRRFQFTQNQKKEDSKTSTSVTSVNQASTSRLEGLOQSENH  
|((signal))|(mature peptide)  
734 747

Score 5.0 at residue 44

Sequence LLLPLAPGAWGWA-RGAPRPPPSPPLSIMGLMPLTKEVAKGSIGRGVLPVELAIEQIRNESL  
|((signal))|(mature peptide)  
31 44

Score 4.9 at residue 497

FIG.3A

SUBSTITUTE SHEET (RULE 26)

5/35

Sequence: ILSALTILGMIMA-SAFLFFNIKNRNQKLIKSSPMNNLIILGGMLSYASIFLFGLDGSFVSE  
|((signal)|((mature peptide))  
484 497  
Score 4.5 at residue 141

Sequence: LMVFGGVCPSVTS-IIAESLQGWNLVQLSFAATTPVLADKKKYPYFFRTVPSDNAVNPAILKLL  
|((signal)|((mature peptide))  
128 141  
Score 4.4 at residue 734

Sequence: FCIVALVIIFCST-ITLCLVFVPKLITLRTNPDAATQNRRFQFTQNQKKEDSKTSTSVTSVNQA  
|((signal)|((mature peptide))  
721 734  
Score 4.1 at residue 165

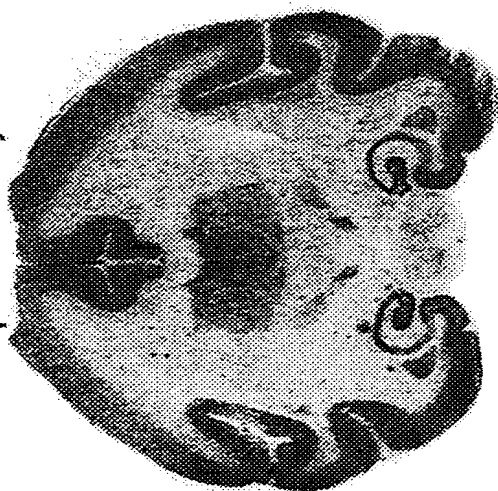
Sequence: VQLSFAATTPVLA-DKKKYPYFFRTVPSDNAVNPAILKLLKHYQWKRVGTLTQDVQRFSEVRND  
|((signal)|((mature peptide))  
152 165  
Score 3.6 at residue 158

Sequence: SLQGWNLVQLSFA-ATTPVLADKKKYPYFFRTVPSDNAVNPAILKLLKHYQWKRVGTLTQDVQR  
|((signal)|((mature peptide))  
145 158

**FIG.3B****SUBSTITUTE SHEET (RULE 26)**

6/35

Distribution of mRNA for EST z43654 in squirrel monkey brain.



at level of thalamus and hippocampus



at level of caudate and putamen



example of cold displayed

at level of cerebellum and occipital cortex

FIG.4

SUBSTITUTE SHEET (RULE 26)

7/35

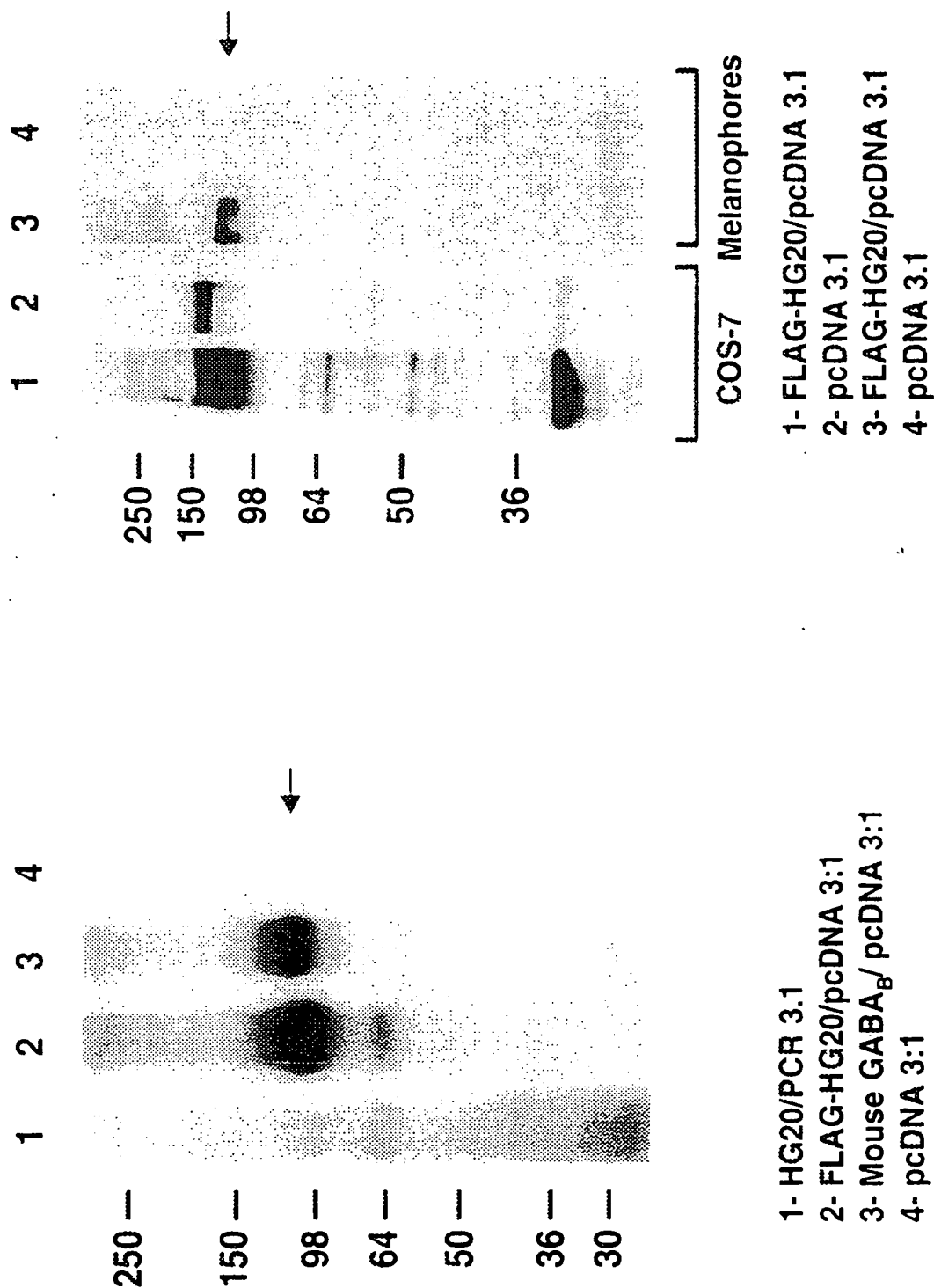


FIG. 5B

FIG. 5A

SUBSTITUTE SHEET (RULE 26)

[illegible]

.....

[illegible]

Number of nodes	Frequency
0	1
1	2
2	3
3	4
4	5
5	6
6	7
7	8
8	9
9	10

FIG. 6

SUBSTITUTE SHEET (RULE 26)



9/35

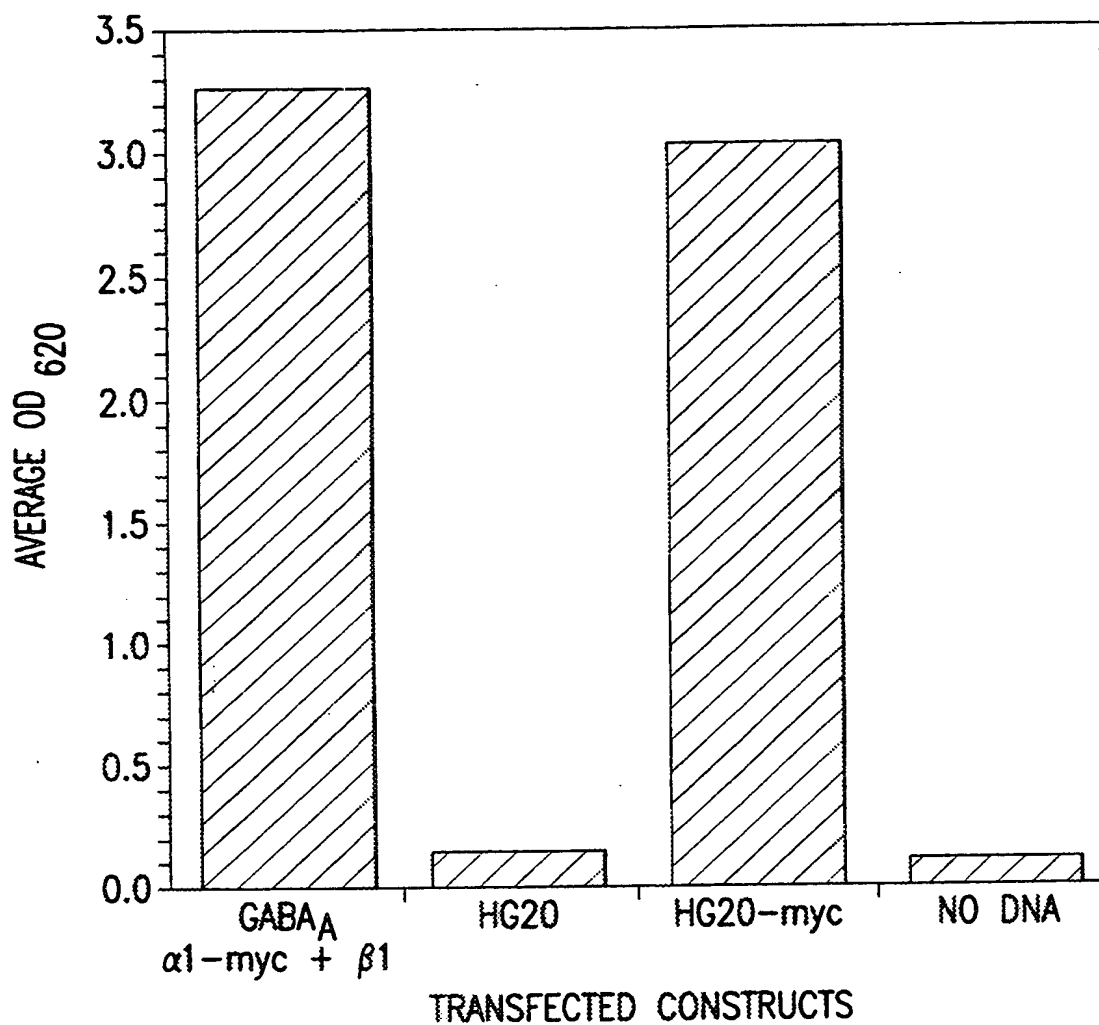


FIG. 7

SUBSTITUTE SHEET (RULE 26)

10/35

GABA-BR1b	69	MGPGGCTPVGWPLPLLLVMAAGVAPVWASHPHL---PRPHPRVPPHPSSERRAVYIGALFPMGGWP-G--GQ
HG20	75	MASPRSSGQGRPPPPPPPARLILLLLPLLLPLAPGAWGARGAPRPPSPPLSIMGLMPLTKEVAKGSIGR
Consensus	75	M.....G.P.P.....A.....L.....R.P.P.....I.L.P.....G.G.
GABA-BR1b	143	ACQPAVEMALEDVNSRRDILPDYELKL IHHDSKCDPGQATKYLYELL YNDPIKIILMPG-CSSVSTLVAEARMW
HG20	149	GVLPAVELAIEQIRN-ESLLRPYFLDLRL YDTECDNAKGLKAFYDAIKYGNHLMVFGGVCPSTSIIESLQGW
Consensus	150	...PAVE.A.E.....L.Y.L.L...D.CD....K.Y.....P.....G.C.SV....AE....W
GABA-BR1b	218	NLIVLSYGSSPALSNRQRFPTFFRTHPSATLHNPTRVKLFEKwGwKKIATIQQTTEVFSTLDDLEERVKEAGI
HG20	224	NLVQLSFAATTPVLADKKKYPYFFRTVPSDNVNPAILKLLKHYYQWKRVTGLTQDVQRFSVRNDLTGVL YGEDI
Consensus	225	NL..LS....P.L....P.FFRT.PS....NP..KL....WK..T.Q....F....DL.....I
GABA-BR1b	290	EITFRQSFSDPAVPVKNLKRQDARIIVGLFYETEARKVFCEVYKERLFGKKYVWFLIGWYADNWF---KTYDPS
HG20	299	EISDTESFSDNPCTSVKCLKGNDVRIILGQFDQDMAAKVFCAYEENMYGSKYQWIIIPGWYEPSWMEQVHTEANS
Consensus	300	EI.....SF..DP...VK.LK...D.RII.G.F...A.KVFC..Y.E...G.KY.W...GWY...W....T....S
GABA-BR1b	365	INCTVEEMTEAVEGHITTEIVMLNPANTRISINMTSQEFVEKLTkRLKRHPETGGFQEAPLAYDAIwALALALN
HG20	372	SRCLRKNLLAAMEGYIGVDFEPLSSKQIKTISGKTPQQY-EREYNN-KRSGVGPSKFHGYAYDGIwIAKTLQRA
Consensus	375	..C.....A.EG.I.....L.....IS..T.Q...E.....KR.....F.....A..L....
GABA-3R1b	440	KTSGGGRSGVRLEDFNYNQTTIDQIYRAMNSSFEFVSGHVVFEDASGSRMAWTLIEQLQGSYKKIGYYDSTK
HG20	446	METLHASSRHQRIDFNFTDHTLGR IILNAMNETNFFGVTGQVVFR-NGERMGTIKFTQFQDSREVKVGEYNAVA
Consensus	450	.....R..DFNY...T...I...AMN..F.GV.G.VVF...G.RM.....Q.Q.....K.G.Y....
GABA-BR1b	512	DDLWSKTDKWIGGSPPADQTLVTKTFRFSQKLFISVSLSLGIWLVVC---LSFNIYNSHVRYIQNSQPNL
HG20	519	DTLEIINDTIRFQGEPPKDKTII--LEQLRKISLPLYSILSALTILGMIMASAFLEFNKRNQKLKIMSSPYM
Consensus	525	D.L.....GS.P.....I.....L.....S.L.S.L.I.....L.FNI.N.....I..S.P...

FIG. 8A

SUBSTITUTE SHEET (RULE 26)

11/35

```

587 GABA-BR1b NNL TAVGCSLALAAVFPLGLDGYHIGRSQFPFVCOARLLWLLGLGSLGYGSMFTKIWWVHTVFTKKEEKEWRKT
591 HG20      NNL IILGMLSYASIFLFGDGSFVSEKTFETLCTVRTIWLTVGYTTAFGAMFAKTWRVHAIF--KNVKMK-KKI
600 Consensus NNL .G.L.A.F.GLDG.....F.C.C.R.W.L.G.....G.MF.K.W.VH..F.K.K.K.....K.

662 GABA-BR1b LEPWKL YATVGLLVGMDVLT LAIWQIVDPLHRTIETFAKEEPKEDIDVSILPQLEHCSSKKMNTWL GIFYGYKGL
666 HG20      IKDQKLLVIVGGMLLIDLCTILICWQAVDPLRRTVEKYSMEPDAGRDISIRPLLEHCENTHMTIWLGI VYAYKGL
675 Consensus . . . . .KL. .VG. . . . .D. . . . .L. .WQ.VDPL. RT. E. . . . .E. . . . .D. SI. P. LEHC. . . . .M. .WLG I. Y. YKGL

737 GABA-BR1b LLLLGI FLAYETKSVSTEKINDHRAVGMAIYNVAVLCLITAPVTMILSSQQDAAFASLAIVFSSYITLVVLFV
741 HG20      LMLFGCFLAWETRNVSI PALNDSKYIGMSVYVNGIMCIIGA AVSFLTRDQPNVQFCIVALV IIFCSTITLCLV FV
750 Consensus L. L. G. FLA. ET. VS. . . . .ND. . . . .GM. YNV. . . . .C. I. A. V. . . . .Q. . . . .F. . . . .L. I. F. S. ITL. . . . .FV

784 GABA-BR1b PKMRRLLITRGE-----W-----QSETQDTMTKGSS-TNNNEEKSR L--LEKE-----NRELEKI-----
816 HG20      PKLITLRTNPDAATQNRRFQFTQNQKEDSKTSTSVTSVNQASTSRLEGLQSENHRLRMKITELDKOLEEVTMQL
825 Consensus PK. . . . .L. T. . . . . . . . . . .Q. . . . .KT. S. T. N. . . . .SRL. L. E. . . . .LE. . . . .

820 GABA-BR1b --IAEKE-----ERVSE-----LRHQLQSRQQLRSRRHPPTPP--DPGG-----
891 HG20      QDTPEKTTYIKQNH YQELNDI LNLGNFTESDGGKAILKNHLDQNPQLQWNTTEPSRTCKDP IEDINSPEHIQRR
900 Consensus . . . . .EK. . . . . . . . . . .L. . . . .L. . . . .QL. . . . .P. . . . .DP. . . . .

844 GABA-BR1b -----LPR-GPSEPPDRLSC-----DGSRVHLL----YK-----
941 HG20      LSLQLPILHHAYLPSIGGV DASCVSPCVSPTASPRHRHVPPSFRVMVSGL
950 Consensus . . . . .LP. G. . . . .C. . . . .R. . . . .

```

FIG.8B

SUBSTITUTE SHEET (RULE 26)

12 / 35

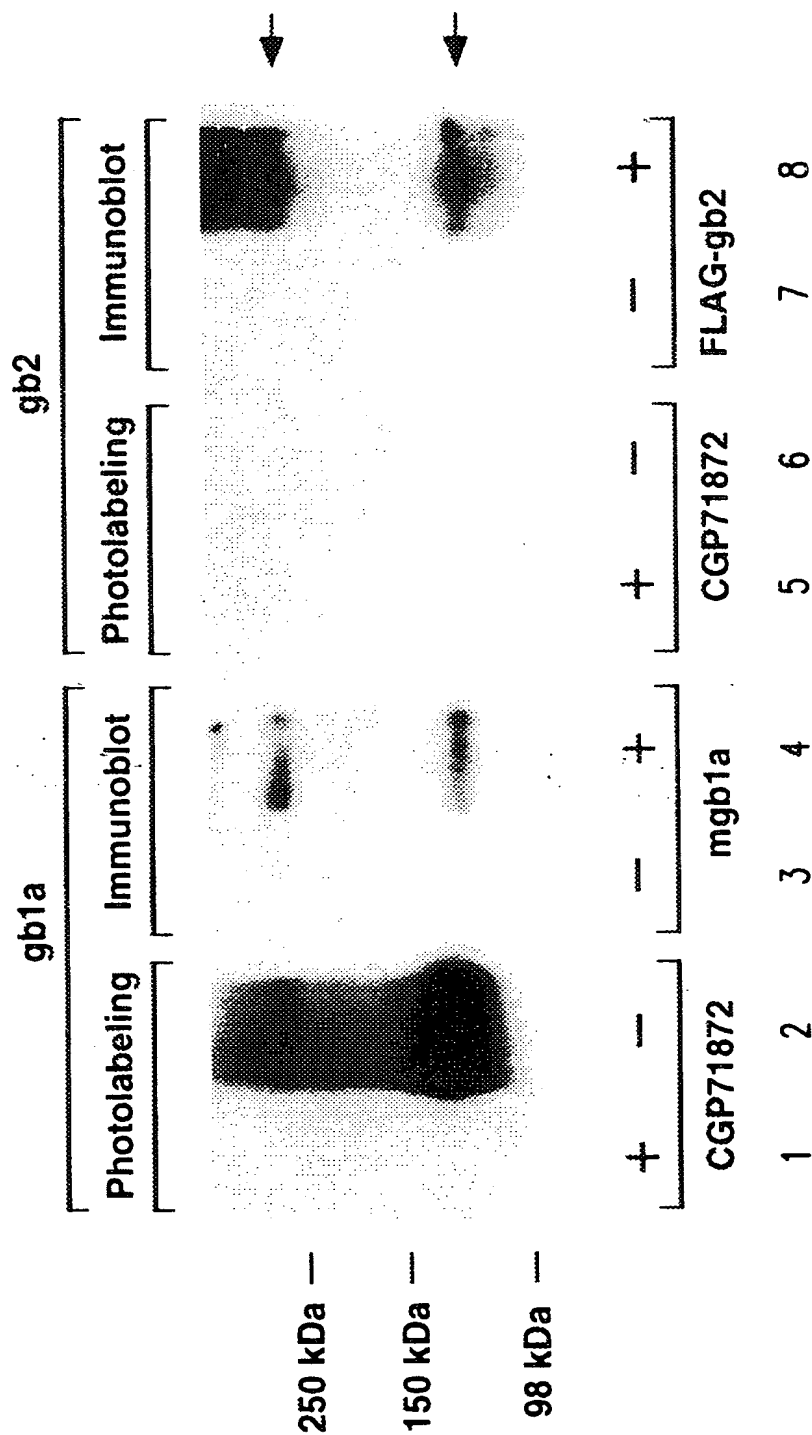


FIG.9

SUBSTITUTE SHEET (RULE 26)

13/35

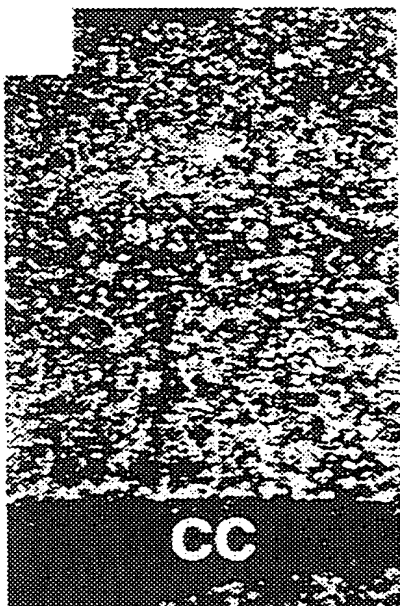


FIG. 10A

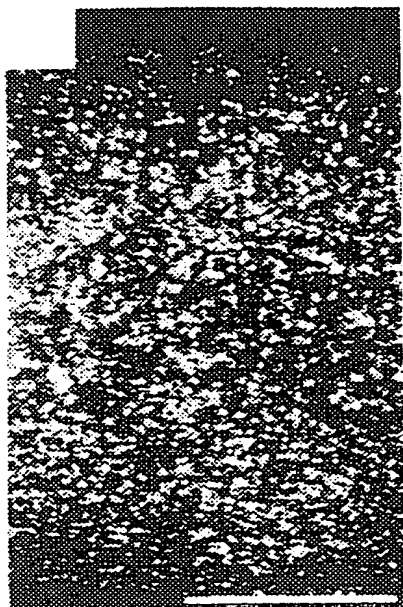


FIG. 10B

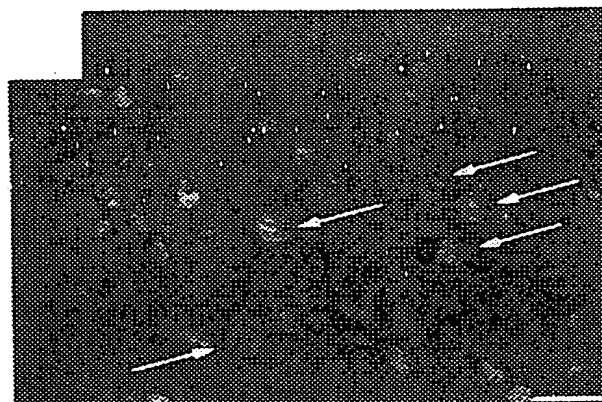


FIG. 10C



FIG. 10D

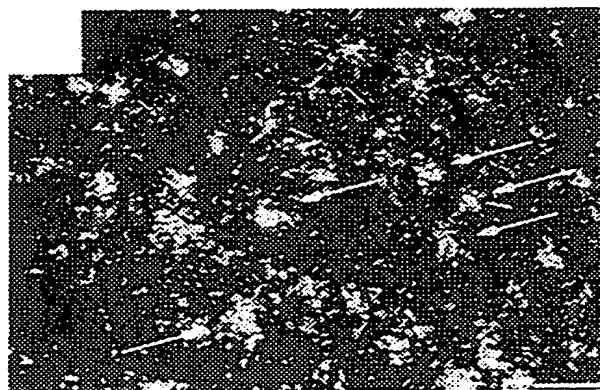


FIG. 10E

SUBSTITUTE SHEET (RULE 26)

14/35

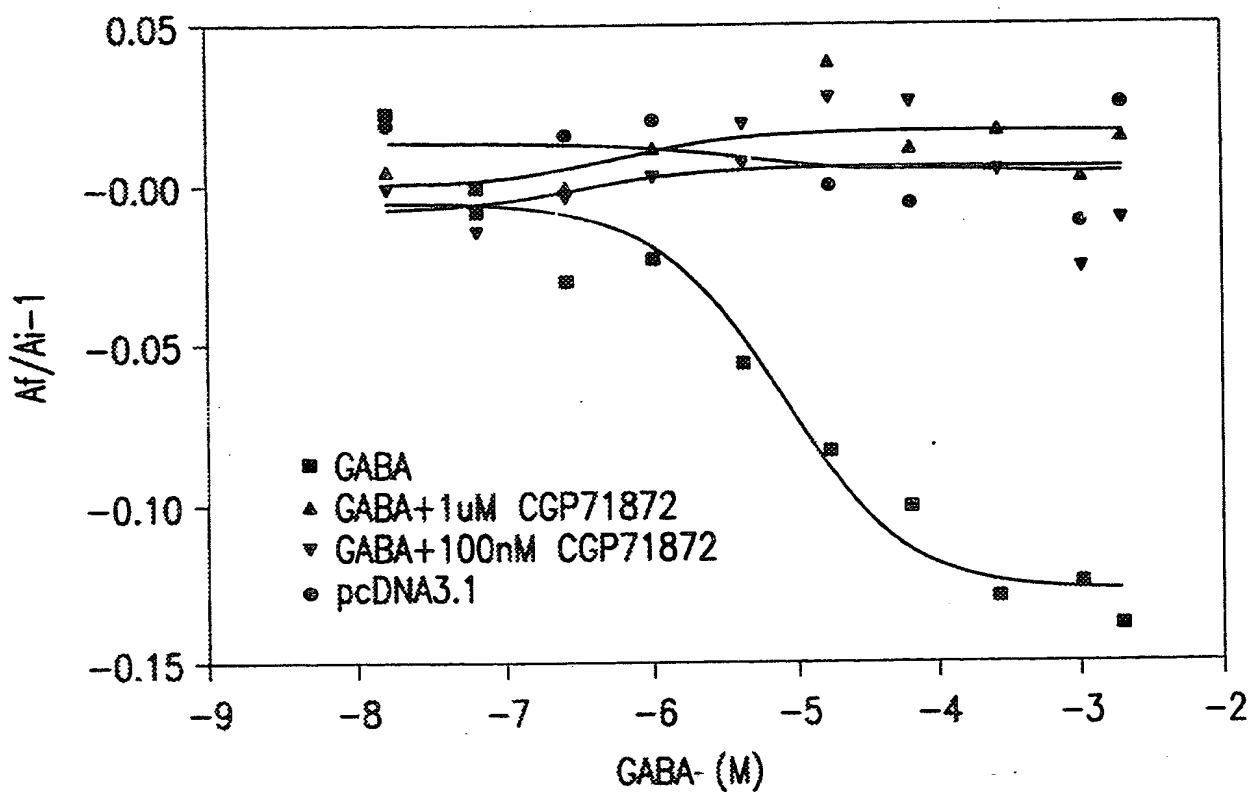


FIG. 11A

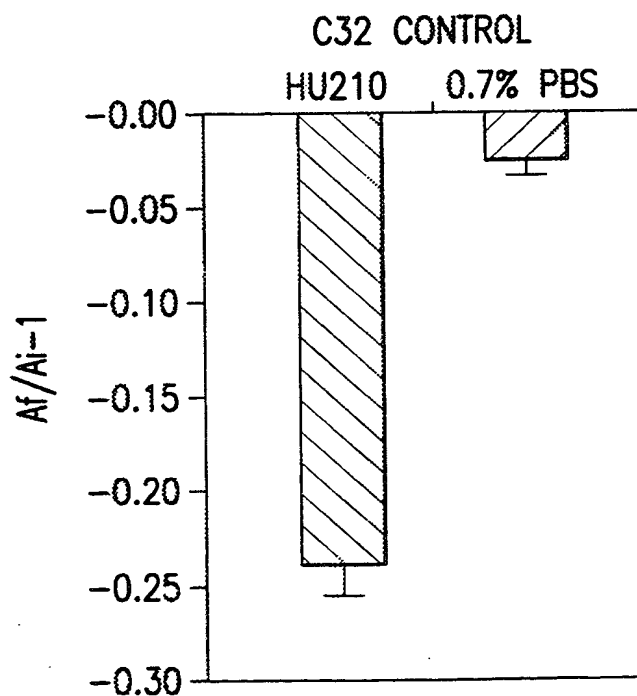


FIG. 11B

SUBSTITUTE SHEET (RULE 26)

15/35

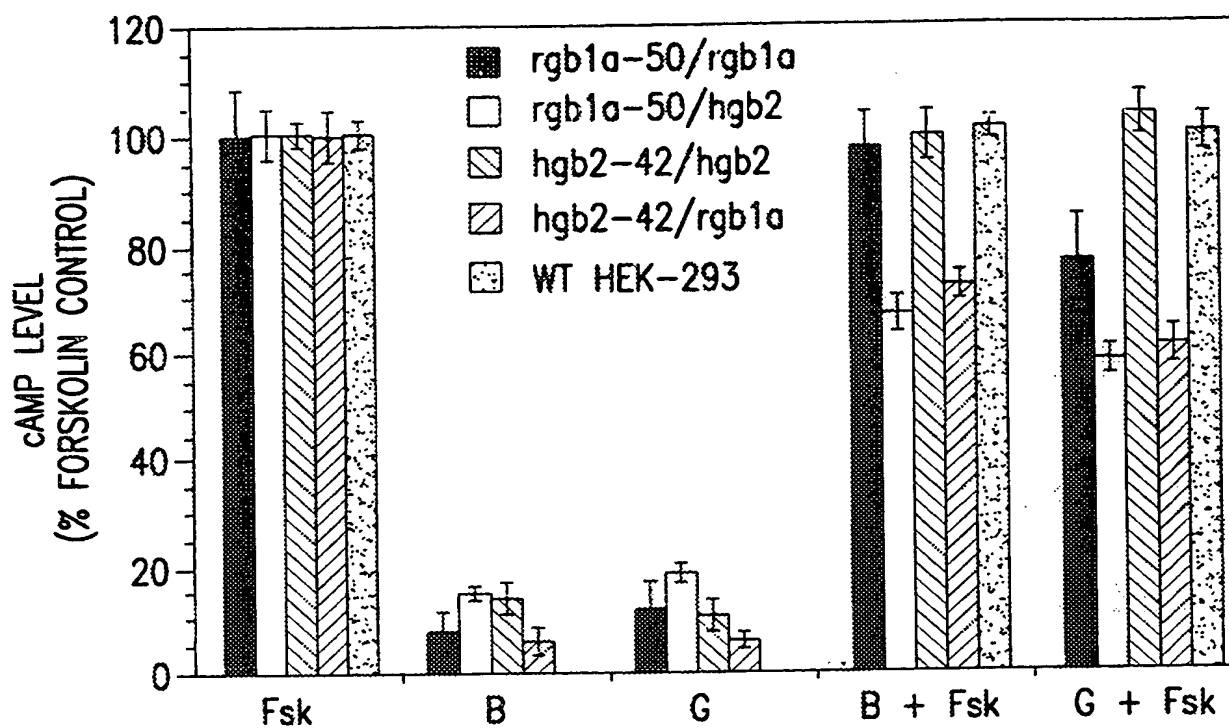


FIG. 12

SUBSTITUTE SHEET (RULE 26)

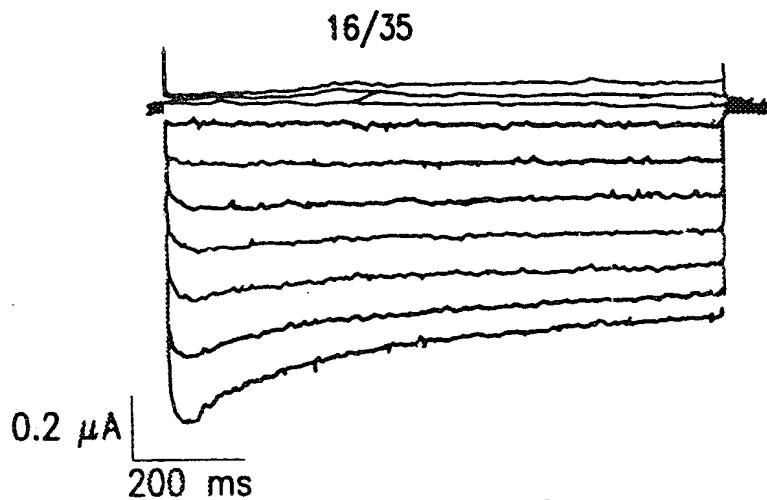


FIG. 13A

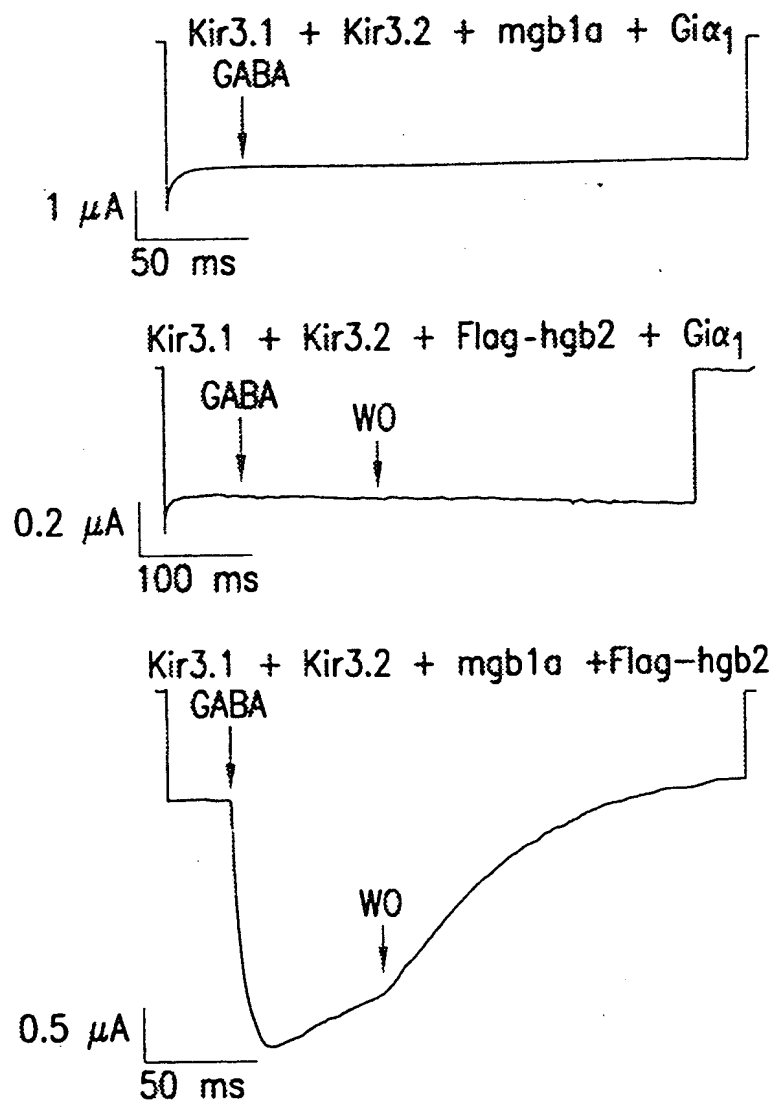


FIG. 13B



17/35

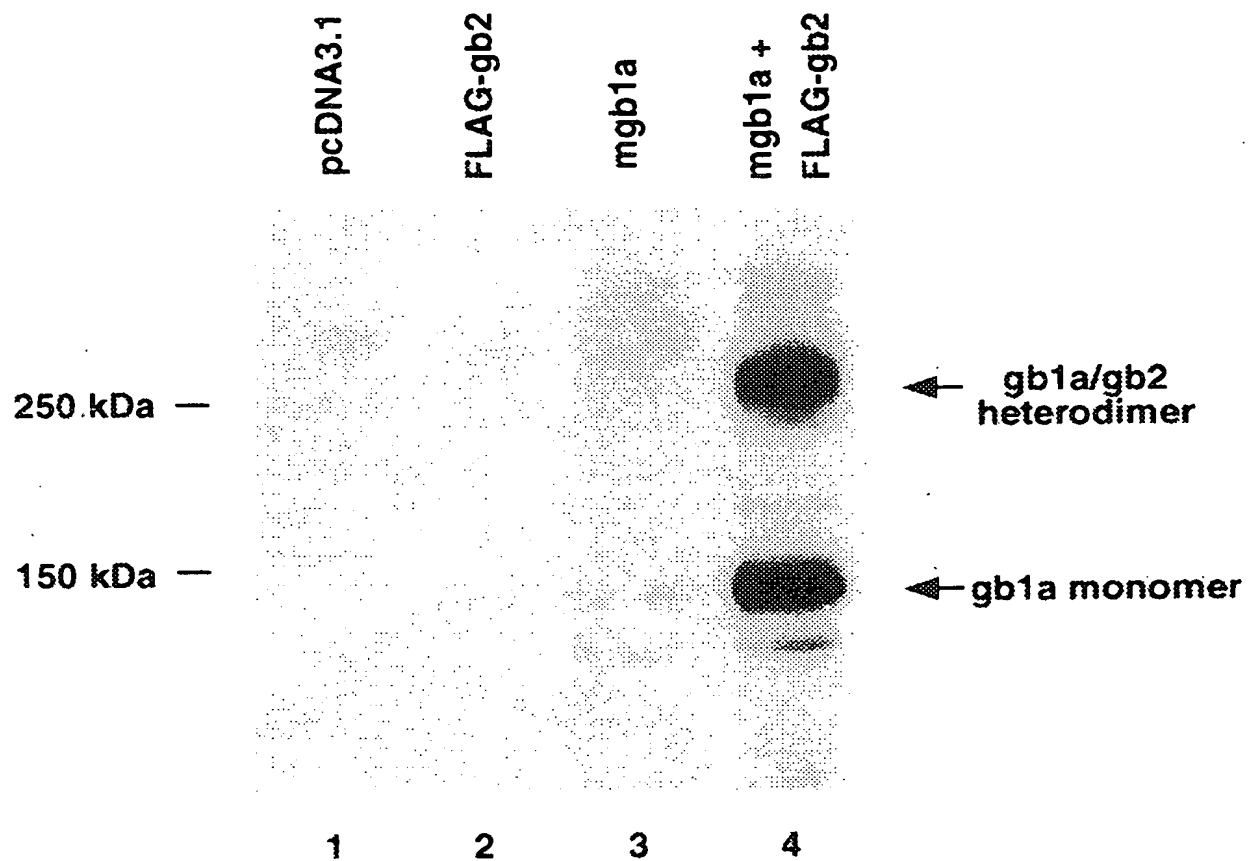


FIG. 14

SUBSTITUTE SHEET (RULE 26)

1 atgctgctgc tgcctgttct gcttctcttc ctccgcccc tgggcgctgg cggggctcag  
61 accccaacg tcacctcgga aggttgccag attatacatc cgccctggga aggtggcatc  
121 aggtaccgtg gcttgattcg cgaccaggtg aaggccatca atttctgcc tgtggactat  
181 gagattgaat atgtgtgccg gggcgaacgc gaggtggtgg ggcccaaggt gcgcaagtgc  
241 ctggccaacg gctcctggac ggatatggac acaccagtc gctgtgtccg aatctgtctc  
301 aagtcttatt tgaccctgga aaatgggaag gttttctga cgggtgggga cctcccagct  
361 ctggatggag cccgggtgga tttccgatgt gacctgact tccatctggt gggcagctcc  
421 cggagcatct gtagtcaggg ccagtggagc accccaagc cccactgcca ggtgaatcga  
481 acgccacact cagaacggcg tgcagtatac atcggggcgc tgtttcccat gagcgggggc  
541 tggccggggg gccaggcctg ccagcctgcg gtggagatgg cgctggagga cgtaacagc  
601 cgcagagaca tcctgccgga ctacgagctc aagcttatcc accacgacag caagtgcgac  
661 ccagggcaag ccaccaagta cttgtatgaa ctactctaca acgaccccat caagatcatc  
721 ctcatgcccg gctgcagctc tgtgtccaca ctggtagccg aggtgcccg gatgtggaac  
781 cttattgtgc tctcatatgg ctccagctca ccagccttgt caaacgaca gcggtttcca  
841 acgttctttc ggacacatcc atccgccaca ctccacaatc ccaccgggt gaaactcttc  
901 gaaaagtggg gctggaagaa gattgccacc atccagcaga ctaccgaggt cttcacctca  
961 acactggatg acctggagga gcgagtgaag gaggtggga ttgagatcac ttttcgacag  
1021 agtttcttct cagatccagc tgtgcctgtt aaaaacctga agcgtcaaga tgctcgaatc  
1081 atcgtgggac ttttctatga gaccgaagcc cggaaagtgt tttgtgaggt ctataaggaa  
1141 cggctctttg ggaagaagta tgtctggttt ctcatcggtt ggtatgctga caactggttc  
1201 aaaacctatg acccgtcaat caattgtaca gtagaagaga tgactgaggc ggtggagggc  
1261 catatcacca cggagattgt catgctgaac cctgccaaca cccgaagcat ttccaacatg  
1321 acatcacagg aatttgtgga gaaactaacc aagcggctga aaagacacc tgaggagact  
1381 ggaggcttcc aggaggcacc actggcctat gatgctatgt gggccttggc tttggccttg  
1441 aacaagacct ctggaggagg tggccgttca ggagtgcgcc tggaggactt taactacaac  
1501 aaccagacca ttacagacca aatctaccgg gccatgaact cctcctcctt tgagggtgtt  
1561 tctggccacg tggcttttga tgccagcggc tcccggatgg catggagcgt tatcagacag  
1621 ctacagggcg gcagctacaa gaagatcggc tactacgaca gcaccaagga tgatctttcc  
1681 tgggtccaaaa cagacaagtg gatcggaggg tctcccccag ccgaccagac cttggtcatc  
1741 aagacattcc gtttctgtc acagaaactc tttatctccg tctcagttct ctccagcctg  
1801 ggcattgttc ttgctgttgt ctgtctgtcc tttaacatct acaactccca cgctcgttat  
1861 atccagaatt cccagcccaa cctgaacaat ctgactgctg tgggtgctc actggcactg  
1921 gctgttgtct tccctctcgg gctggatggt taccacatag ggagaagcca gttcccgttt  
1981 gtctgccagg cccgcctttg gctcttgggc ttgggcttta gtctgggcta tggctctatg  
2041 ttcaccaaga tctggtgggt ccacacagtc ttcacgaaga aggaggagaa gaaggagtgg  
2101 aggaagaccc tagagccctg gaaactctat gccactgtgg gcctgctggt gggcatggat  
2161 gtcctgactc ttgccatctg gcagattgtg gacccttgc accgaaccat tgagactttt  
2221 gccaggagg aaccaaagga agacatcgat gtctccattc tgcccagtt ggagcactgc  
2281 agctccaaga agatgaatac gtggcttggc attttctatg gttacaagg gctgctgctg  
2341 ctgctgggaa tctttcttgc ttacgaaacc aagagcgtgt ccactgaaaa gatcaatgac  
2401 cacaggggcg tgggcatggc tatctacaat gtcgcgttcc tgtgtctcat cactgctcct  
2461 gtgacctga tcctttccag tcagcaggac gcagcctttg cttttgcctc tctggccatc  
2521 gtgttctctt cctacatcac tctggttgtg ctctttgtgc ccaagatgcy caggctgatc  
2581 acccgagggg aatggcagtc tgaaacgcag gacaccatga aaacaggatc atccaccaac  
2641 aacaacgagg aagagaagtc ccgactgttg gagaaggaaa accgagaact ggaaaagatc  
2701 atcgtgaga aagaggagcg cgtctctgaa ctgcgccatc agctccagtc tcggcagcaa  
2761 ctccgctcac ggcgccacc cccaacacc ccagatccct ctgggggcct tcccagggga  
2821 ccctctgagc cccctgaccg gcttagctgt gatgggagtc gagtacattt gctttacaag  
2881 tga

FIG. 15

SUBSTITUTE SHEET (RULE 26)

19/35

MLLLLLLLLLFLRPLGAGGAQTPNVTSEGCQIIHPPWEGGIRYRGLIRDQVKAINFLPVDY  
EIEYVCRGEREVVGPKVRKCLANGSWTDMTPSRCVIRCSKSYLTLENGKVFLTGGDLPA  
LDGARVDFRCDPDFHLVGSSRSICSQGQWSTPKPHCQVNRTPHSERAVYIGALFPMSSG  
WPGGQACQPAVEMALEDVNSRRDILPDYELKLIHDSKCDPGQATKLYELLYNDPIKII  
LMPGCSSVSTLVAEAARMWNLIVLSYGSSSPALSNRQRFPTFFRTHPSATLHNPTRVKLF  
EKWGWKKIATIQQTTEVFTSTLDDLEERVKEAGIEITFRQSFFSDPAVPVKNLKRQDARI  
IVGLFYETEARKVFCEVYKERLFGKKYVWFLIGWYADNWFKTYDPSINCTVEEMTEAVEG  
HITTEIVMLNPANTRSISNMTSQEFVEKLTkRLKRHPREETGGFQEAPLAYDAIWALALAL  
NKTSGGGGRSGVRLEDFNYYNNQITITDQIYRAMNSSSFEGVSGHVVDASGSRMAWTLIEQ  
LQGGSYKKIGYYDSTKDDLWSKTDKWIGGSPADQTLVIKTRFLSQKLFISVSVLSSL  
GIVLAVVCLSFNIYNHARYIQNSQPNLNNLTAVGCSLALAVVFPLGLDGYHIGRSQFPF  
VCQARLWLLGLGFSLGYGSMFTKIWWVHTVFTKKEEKKWRKTLEPWKLYATVGLLVGMD  
VLTIAIWQIVDPLHRTIETFAKEEKPEDIDVSILPQLEHCSSKKMNTWLGIIFYGYKGLLL  
LLGIFLAYETKSVSTEKINDHRAVGMAIYNVAVLCLITAPVTMILSSQDAAFAFASLAI  
VFSSYITLVVLFVPKMRLITRGEWQSETQDTMKTGSSTNNNEEEKSRLLKENRELEKI  
IAEKEERVSELRHQLQSRQQLRSRRHPPTPPDPSGGLPRGPSEPPDRLSCDGSRVHLLYK

FIG. 16

SUBSTITUTE SHEET (RULE 26)

20 / 35

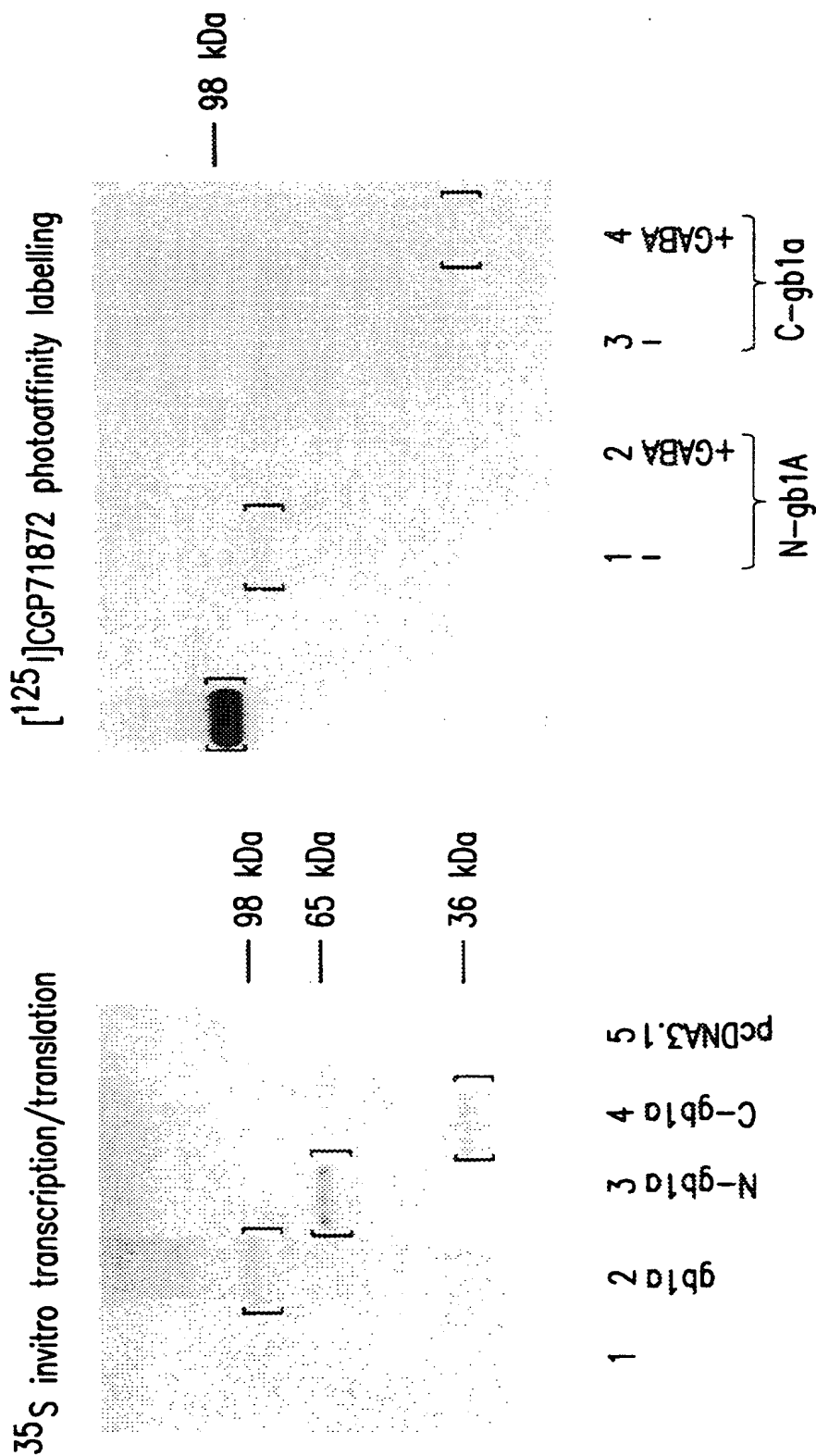


FIG.17A

FIG.17B

21/35

MLLLLLLAPLFLRPPGAGGAHTPNATSEGCQIIHPPWEGGIRYRGLTRDQV  
KAINFLPVDYEIEYVCRGEREVVGPVKRKCLANGSWTDMTPSRCVRICS  
KSYLTLENGKVFLTGGDLPALD GARADFRCDPDFHLVGSSRSICSQGQWST  
PKPHCQVNRTPHSERRAVYIGALFPMSSGGWPGGQACQPAVEMALEDVNS  
RRDILPDYELKLIHHSKCDPGQATKYL YELLYNDPIKIIILMPGCSSVSTLV  
AEAARMWNLIVLSYGSSSPALSNRQRFPTFFRTHPSATLHNPTRVKLFEKW  
GWKKIATIQOTTEVFTSTLDDLEERVKEAGIEITFRQSFFSDPAVPVKNLKRQ  
DARIIVGLFYETEARKVFCEVYKERLFGKKYVWFLIGWYADNWFKIYDPS  
INCTVDEMTEAVEGHITTEIVMLNPANTRSI SNMTSQEFVEKLT KRLKRHPE  
ETGGFQEAPLAYDAIWALALALNKTSGGGGRSGVRLEDNFYNNQTITDQI  
YRAMNSSSFEGVSGHVVDASGSRMAWTLIEQLQGGSYKKIGYYDSTKDD  
LSWSKTDKWIGGSPPADQTLVIKTRFRLSQKLFISVSVLSSLGIVLAVVCLSF  
NIYN SHVRYIQNSQPNLNNLTAVGCSLALAAVFPLGLDGYHIGRNQFPFV  
CQARLWLLGLGFSLGYGSMFTKIWWVHTVFTKKEEKKWRKTLEPWKLY  
ATVGLLVGMDVLT LAIWQIVDPLHRTIETFAKEEPPKEDIDVSILPQLEHCSS  
RKMNTWLGIFYGYKGLLLLLGIFLAYETKSVSTEKINDHRAVGMAIYNVA  
VLCLITAPVTMILSSQDAAFAFASLAIVFSSYITLVVLFVPKMRRLITRGE  
WQSEAQDTMKTGSSTNNNEEEKSRLL EKENRELEKIIAEKEERVSEL RHQLQ  
SRQQLRSRRHPPTPPEPSGGLPRGPPEPPDRLSCDGSRVHLLYK

FIG. 18A

SUBSTITUTE SHEET (RULE 26)

22/35

1 atgttgctgc tgctgctact ggcgccactc ttcctccgcc ccccgggcgc gggcggggcg  
61 catacccccac acgccacctc agaaggttgc cagatcatac acccgccctg ggaagggggc  
121 atcaggtacc ggggcctgac tcgggaccag gtgaaggcta tcaacttcct gccagtggac  
181 tatgagattg agtatgtgtg ccggggggag cgcgaggtgg tggggcccaa ggtccgcaag  
241 tgcctggcca acggctcctg gacagatatg gacacacca gccgctgtgt ccgaatctgc  
301 tccaagtctt atttgaccct ggaaaatggg aaggttttcc tgacgggtgg ggacctccca  
361 gctctggacg gagccccggc ggatttccgg tgtgaccccg acttccatct ggtgggcagc  
421 tcccggagca tctgtagtca gggccagtgg agcaccacca agccccactg ccaggtgaat  
481 cgaacgccac actcagaacg gcgcgcagtg tacatcgggg cactgtttcc catgagcggg  
541 ggctggccag ggggcccaggc ctgccagccc gcggtggaga tggcgtgga ggacgtgaat  
601 agccgcaggg acatcctgcc ggactatgag ctcaagctca tccaccacga cagcaagtgt  
661 gatccaggcc aagccaccaa gtacctatat gagctgctct acaacgaccc tatcaagatc  
721 atccttatgc ctggctgcag ctctgtctcc acgctggtgg ctgaggctgc taggatgtgg  
781 aacctcattg tgctttccta tggctccagc tcaccagccc tgtcaaaccg gcagcgtttc  
841 cccactttct tccgaacgca cccatcagcc acactccaca accctaccgg cgtgaaactc  
901 tttgaaaagt ggggctggaa gaagattgct accatccagc agaccactga ggtcttact  
961 tcgactctgg acgacctgga ggaacgagtg aaggaggctg gaattgagat tactttccgc  
1021 cagagtttct tctcagatcc agctgtgccc gtcaaaaacc tgaagcgcca ggatgcccg  
1081 atcatcgtgg gacttttcta tgagactgaa gcccgaaaag ttttttgtga ggtgtacaag  
1141 gagcgtctct ttgggaagaa gtacgtctgg ttcctcattg ggtggtatgc tgacaattgg  
1201 ttcaagatct acgacccttc tatcaactgc acagtggatg agatgactga ggcggtggag  
1261 ggccacatca caactgagat tgtcatgctg aatcctgcc aatcccgcag cattccaac  
1321 atgacatccc aggaatttgt ggagaaacta accaagcgac tgaaaagaca ccctgaggag  
1381 acaggaggct tccaggaggc accgctggcc tatgatgcca tctgggcctt ggcactggcc  
1441 ctgaacaaga catctggagg aggcggccgt tctggtgtgc gcctggagga cttcaactac  
1501 aacaaccaga ccattācega ccaaactctac cgggcaatga actcttcgtc ctttgaggg  
1561 gtctctggcc atgtggtgtt tgatgccagc ggctctcggg tggcatggac gcttatcgag  
1621 cagcttcagg gtggcagcta caagaagatt ggctactatg acagaccaa ggatgatctt  
1681 tcctggtcca aaacagataa atggattgga gggccccccc cagctgacca gaccctggtc  
1741 atcaagacat tccgcttcct gtcacagaaa ctctttatct ccgtctcagt tctctccagc  
1801 ctgggcattg tcctagctgt tgtctgtctg tcctttaaca tctacaactc acatgtccgt  
1861 tatatccaga actcacagcc caacctgaac aacctgactg ctgtgggctg ctcactggct  
1921 ttagctgctg tcttccccct ggggctcgat ggttaccaca ttgggaggaa ccagtttctt  
1981 ttcgtctgcc agggccgcct ctggctcctg ggcctgggct ttagtctggg ctacggttcc  
2041 atgttcacca agatttgggt ggtccacacg gtcttcacaa agaaggaaga aaagaaggag  
2101 tggaggaaga ctctggaacc ctggaagctg tatgccacag tgggcctgct ggtgggcatg  
2161 gatgtcctca ctctcgccat ctggcagatc gtggaccctc tgcaccggac cattgagaca  
2221 tttgccaaag aggaacctaa ggaagatatt gacgtctcta ttctgcccc gctggagcat  
2281 tgcagctcca ggaagatgaa tacatggctt ggcattttct atggttacaa ggggctgctg  
2341 ctgctgctgg gaatcttctt tgcttatgag accaagagtg tgtccactga gaagatcaat  
2401 gatcaccggg ctgtgggcat ggctatctac aatgtggcag tctgtgcct catcactgct  
2461 cctgtcacca tgattctgtc cagccagcag gatgcagcct ttgcctttgc ctctcttgcc  
2521 atagttttct cctcctatat cactcttggt gtgctctttg tgcccaagat gcgcaggctg  
2581 atcaccgcag gggaatggca gtcggaggcg caggacacca tgaagacagg gtcacgcacc  
2641 aacaacaacg aggaggagaa gtcccggctg ttggagaagg agaaccgtga actggaaaag  
2701 atcattgctg agaaagagga gcgtgtctct gaactgcgcc atcaactcca gtctcggcag  
2761 cagctccgct cccggcgcca cccaccgaca cccccaagaac cctctggggg cctgcccagg  
2821 ggacccccctg agccccccga ccggcttagc tgtgatggga gtcgagtga tttgctttat  
2881 aagtga

FIG. 18B

SUBSTITUTE SHEET (RULE 26)

1 atgctgctgc tgctgctggc gccactcttc ctccgcccc cgggcgcggg cggggcgcag  
61 accccaacg ccacctcaga aggttgccag atcatacacc cgccctggga agggggcatc  
121 aggtaccggg gcctgactcg ggaccagggt aaggctatca acttcctgcc agtggactat  
181 gagattgagt atgtgtgccc gggggagcgc gaggtggtgg ggcccaaggt ccgcaagtgc  
241 ctggccaacg gctcctggac agatatggac acaccagcc gctgtgtccg aatctgtccc  
301 aagtcttatt tgacctgga aaatgggaag gttttcctga cgggtgggga cctcccagct  
361 ctggacggag cccgggtgga tttccggtgt gaccccgact tccatctggt gggcagctcc  
421 cggagcatct gtagtcaggg ccagtggagc accccaagc cccactgcca ggtgaatcga  
481 acgccacact cagaacggcg cgcagtgtac atcggggcac tgtttcccat gagcgggggc  
541 tggccagggg gccaggcctg ccagcccgcg gtggagatgg cgctggagga cgtgaatagc  
601 cgcagggaca tcctgcccga ctatgagctc aagctcatcc accacgacag caagtgtgat  
661 ccaggccaag ccaccaagta cctatatgag ctgctctaca acgaccctat caagatcatc  
721 cttatgcctg gctgcagctc tgtctccacg ctggtggctg aggctgctag gatgtggaac  
781 ctcatgtgtc tttcctatgg ctccagctca ccagccctgt caaacggca gcgtttcccc  
841 actttcttcc gaacgcaccc atcagccaca ctccacaacc ctaccgcgt gaaactcttt  
901 gaaaagtggg gctggaagaa gattgctacc atccagcaga cactgaggt cttcacttcg  
961 actctggacg acctggagga acgagtgaag gaggtggaa ttgagattac tttccgccag  
1021 agtttcttct cagatccagc tgtgcccgtc aaaaacctga agcggcagga tgcccgaatc  
1081 atcgtgggac ttttctatga gactgaagcc cggaaagtgt tttgtgaggt gtacaaggag  
1141 cgtctctttg ggaagaagta cgtctggttc ctcatgggtt ggtatgctga caattggttc  
1201 aagatctacg acccttctat caactgcaca gtggatgaga tgactgaggc ggtggagggc  
1261 cacatcacaa ctgagattgt catgctgaat cctgccaata cccgcagcat ttccaacatg  
1321 acatcccagg aatttgtgga gaaactaacc aagcgactga aaagacaccc tgaggagaca  
1381 ggaggcttcc aggaggcacc gctggcctat gatgccatct gggccttggc actggccctg  
1441 aacaagacat ctggaggagg cggccgttct ggtgtgccc tggaggactt caactacaac  
1501 aaccagacca ttaccgacca aatctaccgg gcaatgaact cttcgtcctt tgagggtgtc  
1561 tctggccatg tgggtgttga tgccagcggc tctcgatgg catggacgct tatcgagcag  
1621 cctcaggggtg gcagctacaa gaagattggc tactatgaca gcaccaagga tgatctttcc  
1681 tgggtccaaaa cagataaatg gattggaggg tccccccag ctgaccagac cctggtcatc  
1741 aagacattcc gcttctgtc acagaaactc tttatctccg tctcagttct ctccagcctg  
1801 ggcattgtcc tagctgttgt ctgtctgtcc tttaacatct acaactcaca tgtccgttat  
1861 atccagaact cacagcccaa cctgaacaac ctgactgctg tgggctgctc actggcttta  
1921 gctgctgtct tccccctggg gctcgatggt taccacattg ggaggaaacca gtttcctttc  
1981 gtctgccagg cncgcctctg gctcctgggc ctgggcttta gtctgggcta cggttccatg  
2041 ttcaccaaga tttggtgggt ccacacgggc ttcacaaaga aggaagaaaa gaaggagtgg  
2101 aggaagactc tggaaccctg gaagctgtat gccacagtgg gcctgctggt gggcatggat  
2161 gtcctcactc tcgccatctg gcagatcgtg gacccctctg accggaccat tgagacattt  
2221 gccaggagg aacctaagga agatattgac gtctctattc tgccccagct ggagcattgc  
2281 agctccagga agatgaatac atggcttggc attttctatg gttacaaggg gctgctgctg  
2341 ctgctgggaa tcttccttgc ttatgagacc aagagtgtgt ccactgagaa gatcaatgat  
2401 caccgggctg tgggcatggc tatctacaat gtggcagtc tgtgcctcat cactgctcct  
2461 gtcaccatga ttctgtccag ccagcaggat gcagcctttg cctttgcctc tcttgccata  
2521 gttttctcct cctatatcac tcttgtttgt ctctttgtgc ccaagatgcg caggctgatc  
2581 acccgagggg aatggcagtc ggaggcgcag gacacatga agacagggtc atcgaccaac  
2641 aacaacgagg aggagaagtc ccggtgttg gagaaggaga accgtgaact ggaaaagatc  
2701 attgctgaga aagaggagcg tgtctctgaa ctgcgccatc aactccagtc tcggcagcag  
2761 ctccgctccc ggcgccaccc accgacaccc ccagaaccct ctgggggcct gccagggga  
2821 cccctgagc cccccgaccg gcttagctgt gatgggagtc gagtgcattt gctttataag  
2881 tgagggtagg gtgaggaggg acaggccagt agggggaggg aaaggagag gggaggggca  
2941 ggggactcag gaagcagggg gtccccatcc ccagctggga agaactgct atccaatctc  
3001 atctcttgta aatacatgtc cccctgtgag ttctgggctg atttggtct ctcatactc  
3061 tgggaaacag acctttttct ctcttactgc ttcagtgaat tttgtatcac ctcttcacaa

FIG. 19A

SUBSTITUTE SHEET (RULE 26)

24/35

3121 tttagttcgt acctggcttg aagctgctca ctgctcacac gctgcctcct cagcagcctc  
3181 actgcatctt tctcttccca tgcaacaccc tcttctagtt accacggcaa cccctgcagc  
3241 tcctctgcct ttgtgctctg ttctgtcca gcaggggtct cccaacaagt gctctttcca  
3301 ccccaaaggg gcctctcctt ttctccactg tcataatctc ttccatctt acttgccctt  
3361 ctatactttc tcacatgtgg ctccccctga attttgcttc ctttgggagc tcattctttt  
3421 cgccaagggt cacatgctcc ttgcctctgc tctgtgcact cacgctcagc acacatgcat  
3481 cctccccctc cctgcgtgtg cccactgaac atgctcatgt gtacacacgc ttttcccgtg  
3541 tgctttcttc atgttcagtc acatgtgctc tcgggtgccc tgcattcaca gctacgtgtg  
3601 cccctctcat ggtcatgggt ctgcccttga gcgtgttttg gtaggcatgt gcaatttgtc  
3661 tagcatgctg agtcatgtct ttctatttg cacacgtcca tgtttatcca tgtactttcc  
3721 ctgtgtaccc tccatgtacc ttgtgtactt tcttccctta aatcatggta ttcttctgac  
3781 agagccatat gtaccctacc ctgcacattg ttatgcactt ttccccaatt catgtttggt  
3841 ggggccatcc acaccctctc cttgtcacag aatctccatt tctgctcaga ttccccccat  
3901 ctccattgca ttcatgtact accctcagtc tacactcaca atcatcttct cccaagactg  
3961 ctcccttttg ttttgtgttt ttttgagggg aattaaggaa aaataagtgg gggcaggttt  
4021 ggagagctgc ttccagtgga tagttgatga gaatcctgac caaaggaagg cacccttgac  
4081 tgttgggata gacagatgga cctatggggg gggaggtggt gtccctttca cactgtggtg  
4141 tctcttgggg aaggatctcc ccgaatctca ataaaccagt gaacagtgtg actcggaaaa  
4201 aaaaaaaaaa aaaaaaaaaa

FIG. 19B



25/35

PROXIMAL TO HSN-1. FCMD, DYS LOCI ON CHROMOSOME 9

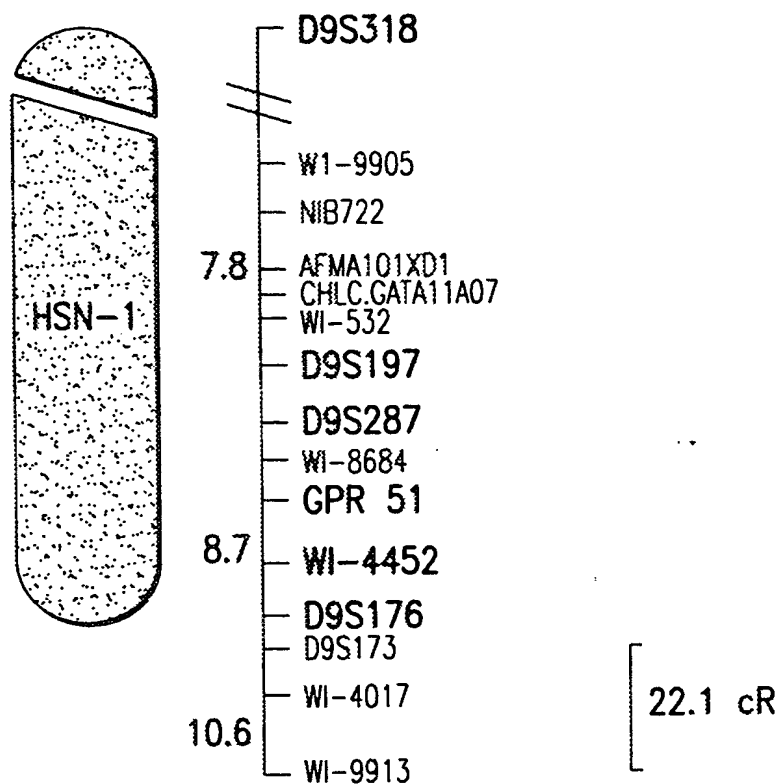


FIG. 20

26/35

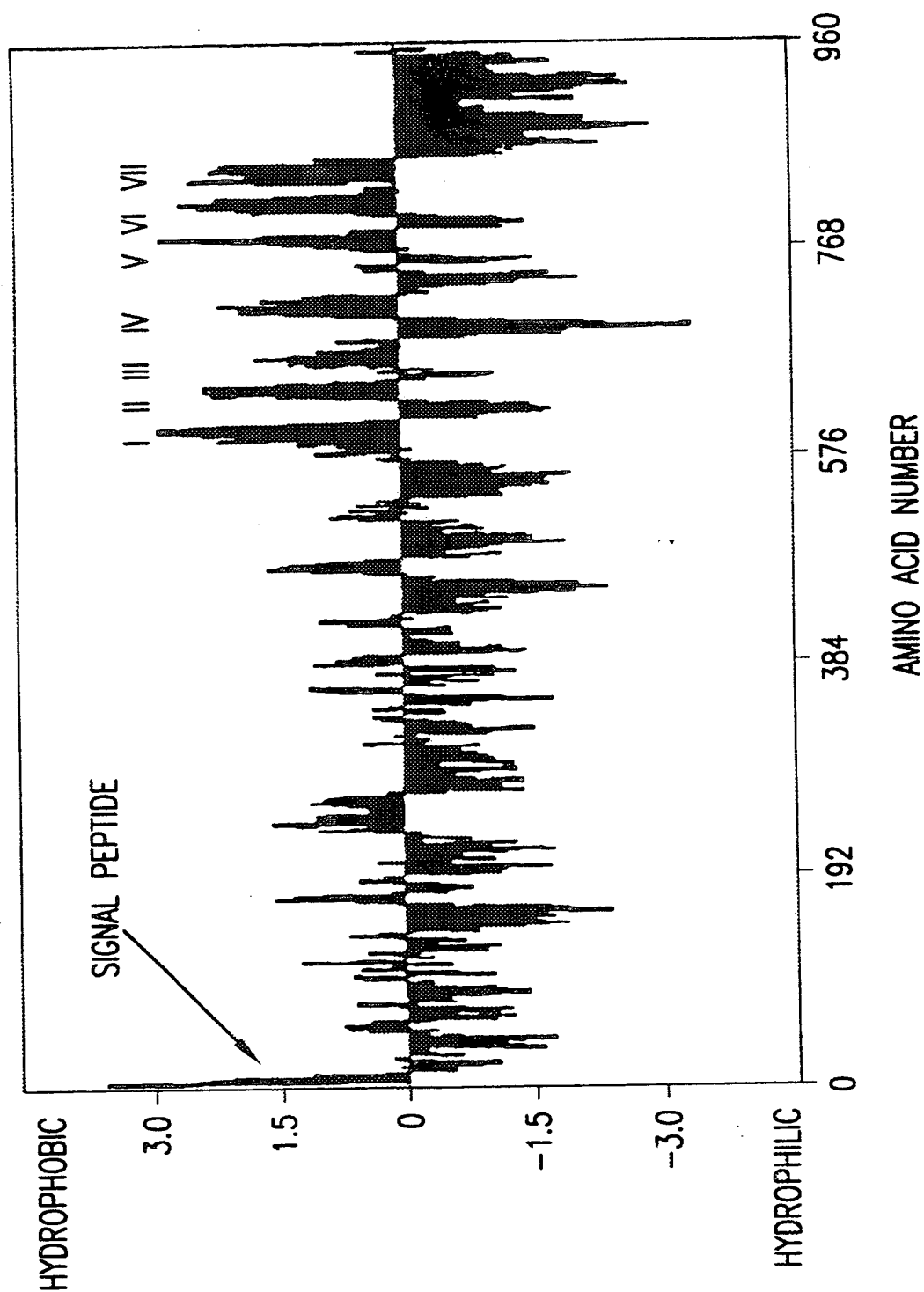


FIG.21

27/35

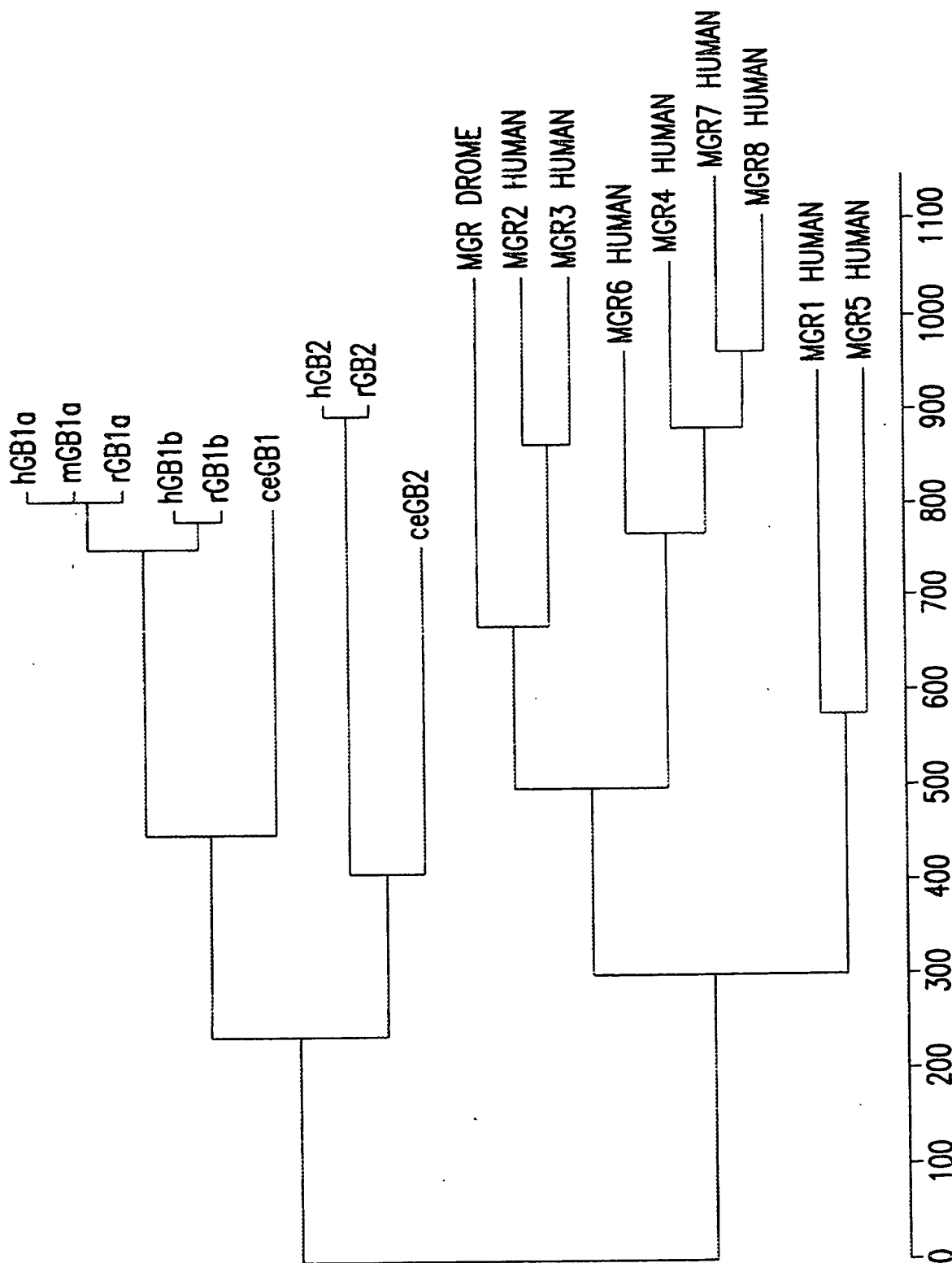


FIG. 22

28/35

COILED-COIL DOMAIN IN C-TERMINUS OF gb1a AND HG20  
MEDIATING HETERODIMERIZATION

gb1 .....WQSEA.QDTMKTGSSSTNNNEEK...SRLEK..ENRELEKIIAEKEERVSELRHQLQSRQQLRRRHPP  
hg20 QNRRFQFTQNKKEDSKTSTSVTSVNQASTSRLEGLQSENHRLRMKII TELDKDLEEVTMQLQDTPEKTTYIKQN

FIG.23

29/35

Human GABA-B1aR  
 C.elegans GABA-B1  
 C.elegans GABA-B2  
 Human GABA-B2

MLLLLLLAPLFLPPPGAGGATPNAISGCGQIHPWPWEGGIRYRGLTRDQVKAINFLPVDVEIEYVCRGEREWGPKVRKCLANGSWTMDMTLSRCVRI<sup>7</sup>CSKSYLTIL  
 -----  
 -----  
 -----  
 -----MASPRSSGQPGPPPPPPPP-----PA

110 120 130 140 150 160 170 180 190 200 210 220

ENGKVF<sup>7</sup>LTGCDLPA<sup>7</sup>LDGAR<sup>7</sup>MD<sup>7</sup>DFRCD<sup>7</sup>DFHLV<sup>7</sup>SSRS<sup>7</sup>IC<sup>7</sup>SG<sup>7</sup>OWSTPKPHCQVNRTPHSERRAVYICALFPM<sup>7</sup>SG<sup>7</sup>W<sup>7</sup>CG<sup>7</sup>AC<sup>7</sup>Q<sup>7</sup>PAVEMAL<sup>7</sup>EDVNSR<sup>7</sup>DTILP<sup>7</sup>VE<sup>7</sup>KL<sup>7</sup>I<sup>7</sup>HDS<sup>7</sup>Q<sup>7</sup>DPGQ<sup>7</sup>  
 -----MFVRSSWILL<sup>7</sup>MG<sup>7</sup>IT<sup>7</sup>IMWASAE<sup>7</sup>DTLH<sup>7</sup>CG<sup>7</sup>TFMES<sup>7</sup>Q<sup>7</sup>-----SG<sup>7</sup>W<sup>7</sup>AG<sup>7</sup>GE<sup>7</sup>AC<sup>7</sup>Q<sup>7</sup>PAVEMAL<sup>7</sup>EDVNSR<sup>7</sup>DTILP<sup>7</sup>VE<sup>7</sup>KL<sup>7</sup>I<sup>7</sup>HDS<sup>7</sup>Q<sup>7</sup>DPGQ<sup>7</sup>  
 -----MNIF<sup>7</sup>RRHG<sup>7</sup>IP<sup>7</sup>PLG<sup>7</sup>VF<sup>7</sup>TVQK<sup>7</sup>-----EGFPD<sup>7</sup>ALP<sup>7</sup>AI<sup>7</sup>RT<sup>7</sup>ALSHVSR<sup>7</sup>SC<sup>7</sup>IL<sup>7</sup>GG<sup>7</sup>YR<sup>7</sup>LEM<sup>7</sup>IVK<sup>7</sup>DT<sup>7</sup>HK<sup>7</sup>TSQ<sup>7</sup>  
 -----LMP<sup>7</sup>L<sup>7</sup>TKEVAK<sup>7</sup>SG<sup>7</sup>IG<sup>7</sup>RG<sup>7</sup>ML<sup>7</sup>PAVEL<sup>7</sup>AI<sup>7</sup>EQ<sup>7</sup>IRNES<sup>7</sup>L<sup>7</sup>RP<sup>7</sup>Y<sup>7</sup>LDLRL<sup>7</sup>YD<sup>7</sup>IE<sup>7</sup>Q<sup>7</sup>DN<sup>7</sup>AK<sup>7</sup>

230 240 250 260 270 280 290 300 310 320 330 340

ATKY<sup>7</sup>VE<sup>7</sup>LYND<sup>7</sup>PIKI<sup>7</sup>IMPG<sup>7</sup>CS<sup>7</sup>VS<sup>7</sup>Q<sup>7</sup>VAEAP<sup>7</sup>RM<sup>7</sup>IN<sup>7</sup>IVLSY<sup>7</sup>SSPAL<sup>7</sup>SNR<sup>7</sup>Q<sup>7</sup>RP<sup>7</sup>PT<sup>7</sup>ERTH<sup>7</sup>PSAT<sup>7</sup>LHNP<sup>7</sup>TR<sup>7</sup>WKL<sup>7</sup>FEK<sup>7</sup>W<sup>7</sup>CK<sup>7</sup>K<sup>7</sup>IAT<sup>7</sup>IQQT<sup>7</sup>IEV<sup>7</sup>FT<sup>7</sup>S<sup>7</sup>IL<sup>7</sup>DDLE<sup>7</sup>ERV<sup>7</sup>KE<sup>7</sup>AG<sup>7</sup>IE<sup>7</sup>IT<sup>7</sup>FR<sup>7</sup>  
 AMQ<sup>7</sup>LD<sup>7</sup>Y<sup>7</sup>Q<sup>7</sup>Y<sup>7</sup>PP<sup>7</sup>IK<sup>7</sup>ML<sup>7</sup>IG<sup>7</sup>CS<sup>7</sup>MT<sup>7</sup>IV<sup>7</sup>IAEAP<sup>7</sup>VM<sup>7</sup>KL<sup>7</sup>MLSY<sup>7</sup>GS<sup>7</sup>SPAL<sup>7</sup>SNR<sup>7</sup>Q<sup>7</sup>RP<sup>7</sup>PT<sup>7</sup>ERTH<sup>7</sup>PSAN<sup>7</sup>Q<sup>7</sup>NP<sup>7</sup>TR<sup>7</sup>I<sup>7</sup>HIME<sup>7</sup>K<sup>7</sup>K<sup>7</sup>W<sup>7</sup>K<sup>7</sup>RF<sup>7</sup>TILMS<sup>7</sup>VE<sup>7</sup>EV<sup>7</sup>FIV<sup>7</sup>IT<sup>7</sup>AK<sup>7</sup>Q<sup>7</sup>LEV<sup>7</sup>SER<sup>7</sup>K<sup>7</sup>KG<sup>7</sup>IK<sup>7</sup>VD<sup>7</sup>RI<sup>7</sup>  
 GKAL<sup>7</sup>FD<sup>7</sup>IASR<sup>7</sup>PR<sup>7</sup>VAI<sup>7</sup>IGG<sup>7</sup>Q<sup>7</sup>TE<sup>7</sup>NE<sup>7</sup>PT<sup>7</sup>AM<sup>7</sup>AL<sup>7</sup>K<sup>7</sup>Y<sup>7</sup>W<sup>7</sup>Q<sup>7</sup>IVQ<sup>7</sup>LSY<sup>7</sup>AE<sup>7</sup>THAM<sup>7</sup>Q<sup>7</sup>QL<sup>7</sup>FT<sup>7</sup>FR<sup>7</sup>MP<sup>7</sup>CS<sup>7</sup>RNT<sup>7</sup>MAK<sup>7</sup>Q<sup>7</sup>VF<sup>7</sup>GN<sup>7</sup>FF<sup>7</sup>GM<sup>7</sup>K<sup>7</sup>RV<sup>7</sup>GT<sup>7</sup>MA<sup>7</sup>Q<sup>7</sup>ND<sup>7</sup>Q<sup>7</sup>-----  
 GLKAF<sup>7</sup>YDA<sup>7</sup>IKY<sup>7</sup>CP<sup>7</sup>NHLMV<sup>7</sup>FG<sup>7</sup>GV<sup>7</sup>Q<sup>7</sup>SV<sup>7</sup>TS<sup>7</sup>ILAE<sup>7</sup>SL<sup>7</sup>QGM<sup>7</sup>LVQ<sup>7</sup>LS<sup>7</sup>FA<sup>7</sup>WT<sup>7</sup>IPVLAD<sup>7</sup>KK<sup>7</sup>YP<sup>7</sup>FE<sup>7</sup>RT<sup>7</sup>VP<sup>7</sup>SD<sup>7</sup>NAV<sup>7</sup>NP<sup>7</sup>IL<sup>7</sup>KL<sup>7</sup>KH<sup>7</sup>YQ<sup>7</sup>MK<sup>7</sup>RV<sup>7</sup>GT<sup>7</sup>IL<sup>7</sup>QDV<sup>7</sup>Q<sup>7</sup>RF<sup>7</sup>SEVR<sup>7</sup>NDL<sup>7</sup>TGVL<sup>7</sup>YGED<sup>7</sup>IE<sup>7</sup>ISDT<sup>7</sup>

350 360 370 380 390 400 410 420 430 440

QSEF<sup>7</sup>SDPAVP<sup>7</sup>V<sup>7</sup>Q<sup>7</sup>RODART<sup>7</sup>IVGL<sup>7</sup>EY<sup>7</sup>ETEAR<sup>7</sup>KV<sup>7</sup>Q<sup>7</sup>EV<sup>7</sup>KER<sup>7</sup>FG<sup>7</sup>KK<sup>7</sup>Y<sup>7</sup>W<sup>7</sup>FL<sup>7</sup>IGWYAD<sup>7</sup>W<sup>7</sup>FK<sup>7</sup>YDP<sup>7</sup>---S<sup>7</sup>IN<sup>7</sup>CT<sup>7</sup>VD<sup>7</sup>EMTEA<sup>7</sup>VECH<sup>7</sup>ITTE<sup>7</sup>IM<sup>7</sup>IN<sup>7</sup>PAN<sup>7</sup>-----TRS<sup>7</sup>SM<sup>7</sup>TSQE<sup>7</sup>VEK<sup>7</sup>  
 QSFY<sup>7</sup>CD<sup>7</sup>PTDAM<sup>7</sup>Q<sup>7</sup>RODART<sup>7</sup>IVGL<sup>7</sup>FY<sup>7</sup>TEAR<sup>7</sup>KV<sup>7</sup>Q<sup>7</sup>Q<sup>7</sup>AM<sup>7</sup>H<sup>7</sup>Q<sup>7</sup>Y<sup>7</sup>Q<sup>7</sup>RRY<sup>7</sup>W<sup>7</sup>FF<sup>7</sup>IGWYAD<sup>7</sup>W<sup>7</sup>Y<sup>7</sup>IPPP<sup>7</sup>---EEH<sup>7</sup>LN<sup>7</sup>CT<sup>7</sup>AE<sup>7</sup>Q<sup>7</sup>MT<sup>7</sup>EA<sup>7</sup>VE<sup>7</sup>FT<sup>7</sup>TES<sup>7</sup>ML<sup>7</sup>SRON<sup>7</sup>-----IPAL<sup>7</sup>SEM<sup>7</sup>IG<sup>7</sup>MO<sup>7</sup>Q<sup>7</sup>Q<sup>7</sup>OR<sup>7</sup>  
 -----PRYAL<sup>7</sup>VRG<sup>7</sup>VRT<sup>7</sup>ILVD<sup>7</sup>VE<sup>7</sup>EMAA<sup>7</sup>IT<sup>7</sup>LDAG<sup>7</sup>TH<sup>7</sup>RG<sup>7</sup>AY<sup>7</sup>GD<sup>7</sup>MM<sup>7</sup>W<sup>7</sup>IL<sup>7</sup>PGY<sup>7</sup>HSD<sup>7</sup>RM<sup>7</sup>LN<sup>7</sup>Q<sup>7</sup>-----THDN<sup>7</sup>CT<sup>7</sup>VE<sup>7</sup>EM<sup>7</sup>RE<sup>7</sup>AK<sup>7</sup>NH<sup>7</sup>SV<sup>7</sup>EF<sup>7</sup>AL<sup>7</sup>TRRD<sup>7</sup>VD<sup>7</sup>TK<sup>7</sup>IVGN<sup>7</sup>IT<sup>7</sup>SPY<sup>7</sup>VT<sup>7</sup>LN<sup>7</sup>LF<sup>7</sup>Q<sup>7</sup>R<sup>7</sup>  
 ESFSND<sup>7</sup>PCT<sup>7</sup>SVKK<sup>7</sup>KL<sup>7</sup>KG<sup>7</sup>Q<sup>7</sup>VRT<sup>7</sup>IL<sup>7</sup>GG<sup>7</sup>TF<sup>7</sup>DON<sup>7</sup>MAAK<sup>7</sup>VF<sup>7</sup>Q<sup>7</sup>CA<sup>7</sup>Y<sup>7</sup>EE<sup>7</sup>MY<sup>7</sup>GS<sup>7</sup>K<sup>7</sup>Q<sup>7</sup>W<sup>7</sup>IL<sup>7</sup>PGWYEP<sup>7</sup>SW<sup>7</sup>WE<sup>7</sup>Q<sup>7</sup>HT<sup>7</sup>EANS<sup>7</sup>SR<sup>7</sup>Q<sup>7</sup>RK<sup>7</sup>NLL<sup>7</sup>AM<sup>7</sup>EGY<sup>7</sup>IG<sup>7</sup>Q<sup>7</sup>DE<sup>7</sup>PL<sup>7</sup>SSK<sup>7</sup>Q<sup>7</sup>-----IK<sup>7</sup>TI<sup>7</sup>SG<sup>7</sup>K<sup>7</sup>TP<sup>7</sup>QQ<sup>7</sup>YERE<sup>7</sup>

450 460 470 480 490 500 510 520 530 540 550 560

LT<sup>7</sup>KRL<sup>7</sup>KR<sup>7</sup>HP<sup>7</sup>EE<sup>7</sup>TGG<sup>7</sup>FE<sup>7</sup>APLAYDA<sup>7</sup>IMWAL<sup>7</sup>AL<sup>7</sup>IM<sup>7</sup>Q<sup>7</sup>SGGCG<sup>7</sup>RS<sup>7</sup>--GV<sup>7</sup>RL<sup>7</sup>ED<sup>7</sup>EN<sup>7</sup>Q<sup>7</sup>NT<sup>7</sup>TD<sup>7</sup>Q<sup>7</sup>IYRAM<sup>7</sup>NS<sup>7</sup>FE<sup>7</sup>GV<sup>7</sup>SC<sup>7</sup>W<sup>7</sup>AF<sup>7</sup>DA<sup>7</sup>SG<sup>7</sup>SR<sup>7</sup>W<sup>7</sup>AM<sup>7</sup>IL<sup>7</sup>TEQ<sup>7</sup>GG<sup>7</sup>SY<sup>7</sup>K<sup>7</sup>IGY<sup>7</sup>YD<sup>7</sup>ST<sup>7</sup>KOD<sup>7</sup>IS<sup>7</sup>MSK<sup>7</sup>  
 LT<sup>7</sup>QYF<sup>7</sup>QK<sup>7</sup>DTAN<sup>7</sup>CG<sup>7</sup>PE<sup>7</sup>APLAYDA<sup>7</sup>WAL<sup>7</sup>AL<sup>7</sup>AF<sup>7</sup>NC<sup>7</sup>TRNN<sup>7</sup>-LPS<sup>7</sup>---HIR<sup>7</sup>LEN<sup>7</sup>TD<sup>7</sup>KN<sup>7</sup>Q<sup>7</sup>W<sup>7</sup>AD<sup>7</sup>IL<sup>7</sup>FCQ<sup>7</sup>VKN<sup>7</sup>TS<sup>7</sup>FR<sup>7</sup>GV<sup>7</sup>SK<sup>7</sup>W<sup>7</sup>AF<sup>7</sup>SD<sup>7</sup>SG<sup>7</sup>RI<sup>7</sup>AF<sup>7</sup>TD<sup>7</sup>TEQ<sup>7</sup>GG<sup>7</sup>Q<sup>7</sup>Y<sup>7</sup>IM<sup>7</sup>GY<sup>7</sup>DT<sup>7</sup>TS<sup>7</sup>CD<sup>7</sup>LE<sup>7</sup>W<sup>7</sup>YN<sup>7</sup>  
 AGDVWNE<sup>7</sup>ITQL<sup>7</sup>DP<sup>7</sup>NT<sup>7</sup>WR<sup>7</sup>CV<sup>7</sup>LYDGL<sup>7</sup>MT<sup>7</sup>L<sup>7</sup>AL<sup>7</sup>SH<sup>7</sup>SG<sup>7</sup>Q<sup>7</sup>-----NAE<sup>7</sup>FSHK<sup>7</sup>ME<sup>7</sup>AI<sup>7</sup>ONS<sup>7</sup>FC<sup>7</sup>QL<sup>7</sup>TC<sup>7</sup>K<sup>7</sup>Y<sup>7</sup>AF<sup>7</sup>ANN<sup>7</sup>-FR<sup>7</sup>GL<sup>7</sup>VD<sup>7</sup>IK<sup>7</sup>MS<sup>7</sup>Q<sup>7</sup>Q<sup>7</sup>W<sup>7</sup>PF<sup>7</sup>AV<sup>7</sup>TC<sup>7</sup>--ADDE<sup>7</sup>FK<sup>7</sup>I<sup>7</sup>  
 YNNKR<sup>7</sup>---SG<sup>7</sup>VC<sup>7</sup>PS<sup>7</sup>-KF<sup>7</sup>H<sup>7</sup>GV<sup>7</sup>AY<sup>7</sup>DG<sup>7</sup>IMV<sup>7</sup>IAK<sup>7</sup>TLORAME<sup>7</sup>TL<sup>7</sup>HASSR<sup>7</sup>QR<sup>7</sup>IQDF<sup>7</sup>NT<sup>7</sup>DHT<sup>7</sup>LC<sup>7</sup>RI<sup>7</sup>IL<sup>7</sup>NAME<sup>7</sup>TNE<sup>7</sup>FG<sup>7</sup>VT<sup>7</sup>Q<sup>7</sup>Q<sup>7</sup>W<sup>7</sup>FR<sup>7</sup>NG<sup>7</sup>-ER<sup>7</sup>MG<sup>7</sup>TK<sup>7</sup>IF<sup>7</sup>Q<sup>7</sup>Q<sup>7</sup>SR<sup>7</sup>EM<sup>7</sup>KV<sup>7</sup>GED<sup>7</sup>NAVAD<sup>7</sup>TL<sup>7</sup>EL<sup>7</sup>IN<sup>7</sup>

FIG. 24A

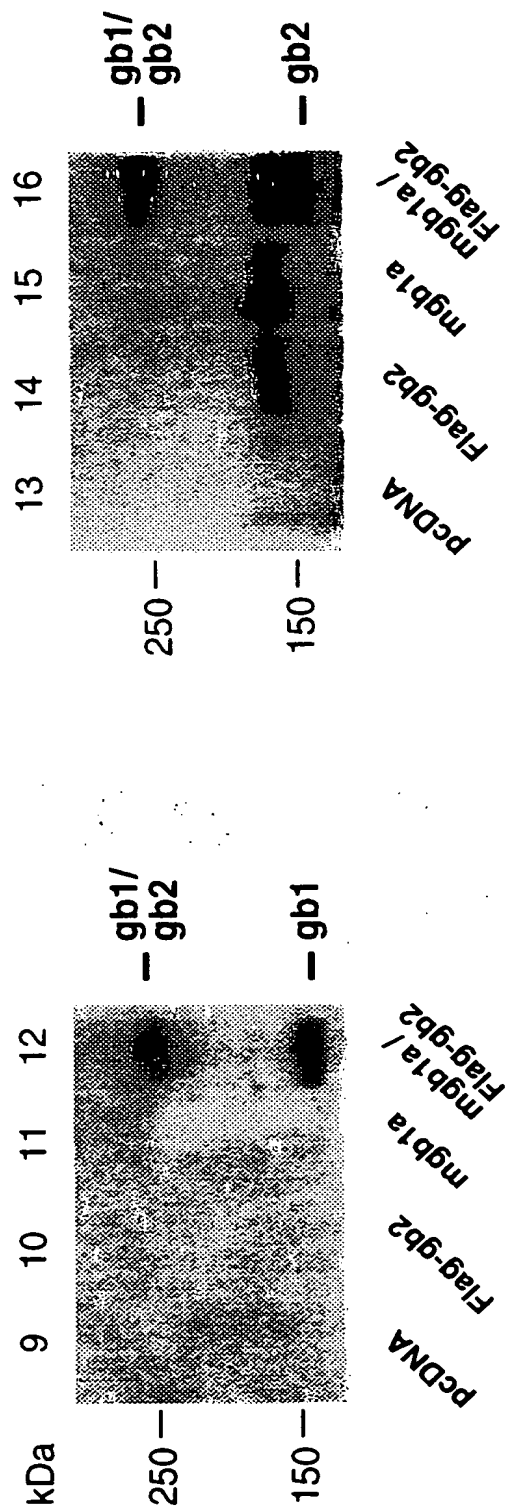
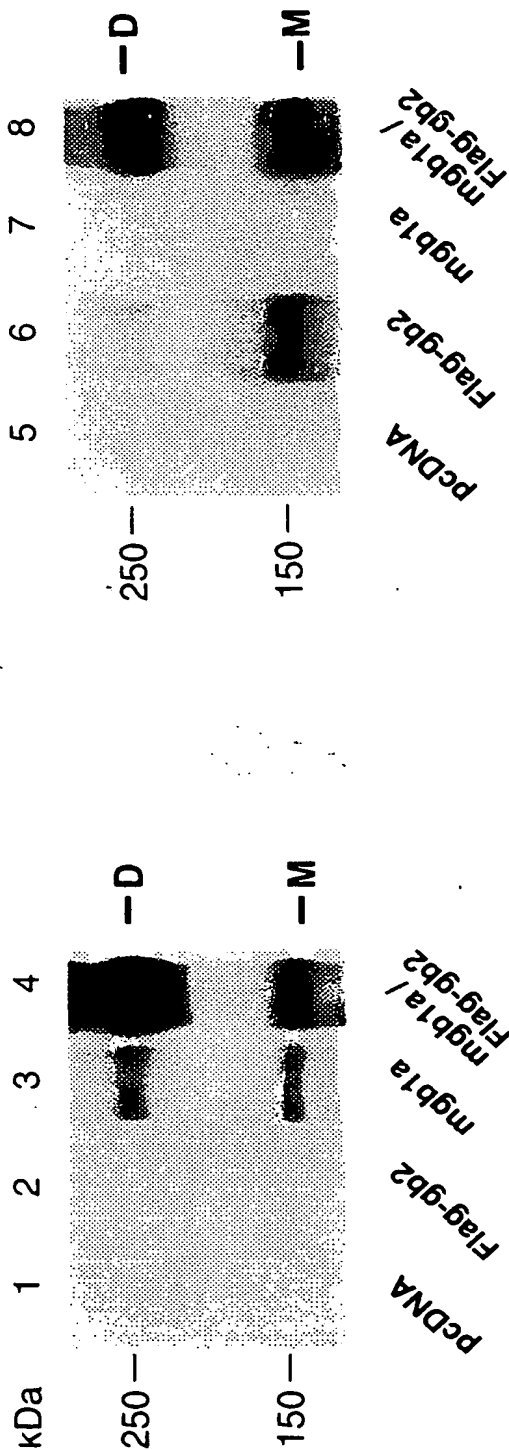
[illegible]

PLRMKITELDKDL EEVTMLQDITPEKTTYIKQNIHYQELNDJLNLGNFTSTGGKAILKNHLDQNPQLQWNTTEPSRTCKDPDIEDINSPEHIQRRLSLQLPLIHHAYLPSICGVDS

**FIG. 24B**

CVSPCVSPTASPRHRHVPPSFRVMVSGL

31 / 35



mGABab1a M L L L L L L L L L F L R P L G A G G A Q T P N V T S E G C G I I H P P W E G G I R Y R G L I R D Q V K A I N F L P V D Y E I E Y V C R G E R E W G P K V R K C L A N G S W I T D M D I P S R C V R I C S Y I T I L E N G K V F L T G G D L P : 119  
 hGABab1a M L L L L L L L L L F L R P P G A G G A Q T P N A T S E G C G I I H P P W E G G I R Y R G L I R D Q V K A I N F L P V D Y E I E Y V C R G E R E W G P K V R K C L A N G S W I T D M D I P S R C V R I C S Y I T I L E N G K V F L T G G D L P : 120  
 hGABab2 M L L L L L L L L L F L R P P G A G G A Q T P N A T S E G C G I I H P P W E G G I R Y R G L I R D Q V K A I N F L P V D Y E I E Y V C R G E R E W G P K V R K C L A N G S W I T D M D I P S R C V R I C S Y I T I L E N G K V F L T G G D L P : 30  
 mGluR1 M L L L L L L L L L F L R P P G A G G A Q T P N A T S E G C G I I H P P W E G G I R Y R G L I R D Q V K A I N F L P V D Y E I E Y V C R G E R E W G P K V R K C L A N G S W I T D M D I P S R C V R I C S Y I T I L E N G K V F L T G G D L P : 44  
 LivK M L L L L L L L L L F L R P P G A G G A Q T P N A T S E G C G I I H P P W E G G I R Y R G L I R D Q V K A I N F L P V D Y E I E Y V C R G E R E W G P K V R K C L A N G S W I T D M D I P S R C V R I C S Y I T I L E N G K V F L T G G D L P : 29  
 LivBP M L L L L L L L L L F L R P P G A G G A Q T P N A T S E G C G I I H P P W E G G I R Y R G L I R D Q V K A I N F L P V D Y E I E Y V C R G E R E W G P K V R K C L A N G S W I T D M D I P S R C V R I C S Y I T I L E N G K V F L T G G D L P : 6  
 mGABab1a A L D G A R V D F R C D P D F H L V G S S R S I C S Q C G N S T P K P H C Q W R T P H S E R R A V Y I G A L F P M S . G G W P G . G Q A C O P A V E M A L E D V N S R R D . I L P D Y E L K L I H H D . S K D P Q A T K Y L Y E L L Y : 233  
 hGABab1a A L D G A R V D F R C D P D F H L V G S S R S I C S Q C G N S T P K P H C Q W R T P H S E R R A V Y I G A L F P M S . G G W P G . G Q A C O P A V E M A L E D V N S R R D . I L P D Y E L K L I H H D . S K D P Q A T K Y L Y E L L Y : 234  
 hGABab2 L L . . . . . L P L A P G A W G A . . . . . R Q A P R P . . . . . P P S S P . P L S I M G L M P L T K E V A K G S I G R G V L P A V E L A I E Q I . R N E S L L R P Y F L D L R L Y D . T E C D N A K G L K A F Y D A I K : 122  
 mGluR1 V I I G A L F S V H H O P P A E K V P E R K C G E I R E Q C G I Q R . . . . . V E A M F H T L D K I N A D P V L L P N I T L G S E I . I R D . S O W H S S V A L E Q S I E F I R D S L I S I R D E K D G I . . . . . N R C L P : 142  
 LivK V V G A M . . . . . S G P I . A Q W G I M E . . . . . F N G A E Q A I K D I N A K G G I K G D K L V G V E . . . . . Y D D . A C . . . . . D P K Q A V A V A N K I V N : 90  
 LivBP V V G A M . . . . . S G P V . A Q W G D O E . . . . . F T C A E Q A V A D I N A K G G I K G N K L Q I A K . . . . . Y D D . A C . . . . . D P K Q A V A V A N K V V N : 67  
 mGABab1a P I K I I L M P G C S S V S I L V A E A A R M M N . . . . . L I V L S Y G S S P A L S I R P R F P I F R I L P S A T I L H N P T R V K L F E . . . . . K W G M K K I A T I Q Q T T E V F T S . T I D D L E E R V K E A G I : 334  
 hGABab1a P I K I I L M P G C S S V S I L V A E A A R M M N . . . . . L I V L S Y G S S P A L S I R P R F P I F R I L P S A T I L H N P T R V K L F E . . . . . K W G M K K I A T I Q Q T T E V F T S . T I D D L E E R V K E A G I : 335  
 hGABab2 P N H L M V F G V G P S V I S I V A E S L Q W N . . . . . L V Q L S F A A T T E M A D K K K P Y F F R T P S D N A V N P A I L K L I K . . . . . H Y Q M K R V G T L T O D V Q R F S E . . . . . V R N D L T G V L Y G E D I : 224  
 mGluR1 D Q S L P P G R T K K P I A G V I . P G C S S V A I Q V Q N L L Q L F D . . . . . I P Q I A Y S A T S I D S K T L Y K Y E L R V P S D T L Q . . . . . A R A M D I V K R Y N M T Y V S A V . H T E G N Y G E S G M D A F K E L A Q E Q L : 253  
 LivK I K Y V I . G H L C S S I S T O P A S . . . . . D I Y E D E G I L M I S P G A T A P E L I T A G C . Y Q H I M R I A G L D S S Q P T A A N Y I L E T V K P . Q R . . . . . I A I I . H D K Q Q Y Q E . G L A R . . . . . S V Q D G L : 185  
 LivBP I K Y V I . G H L C S S I S T O P A S . . . . . D I Y E D E G I L M I T P A T A P E L I T A G C . Y Q L I U R I T G L D S Q Q P T A A N Y I L E K V K P . Q R . . . . . I A I V . H D K Q Q Y Q E . G L A R . . . . . A V Q D G L : 162

FIG.26A-1



33/35

FIG. 26A-2

34/35

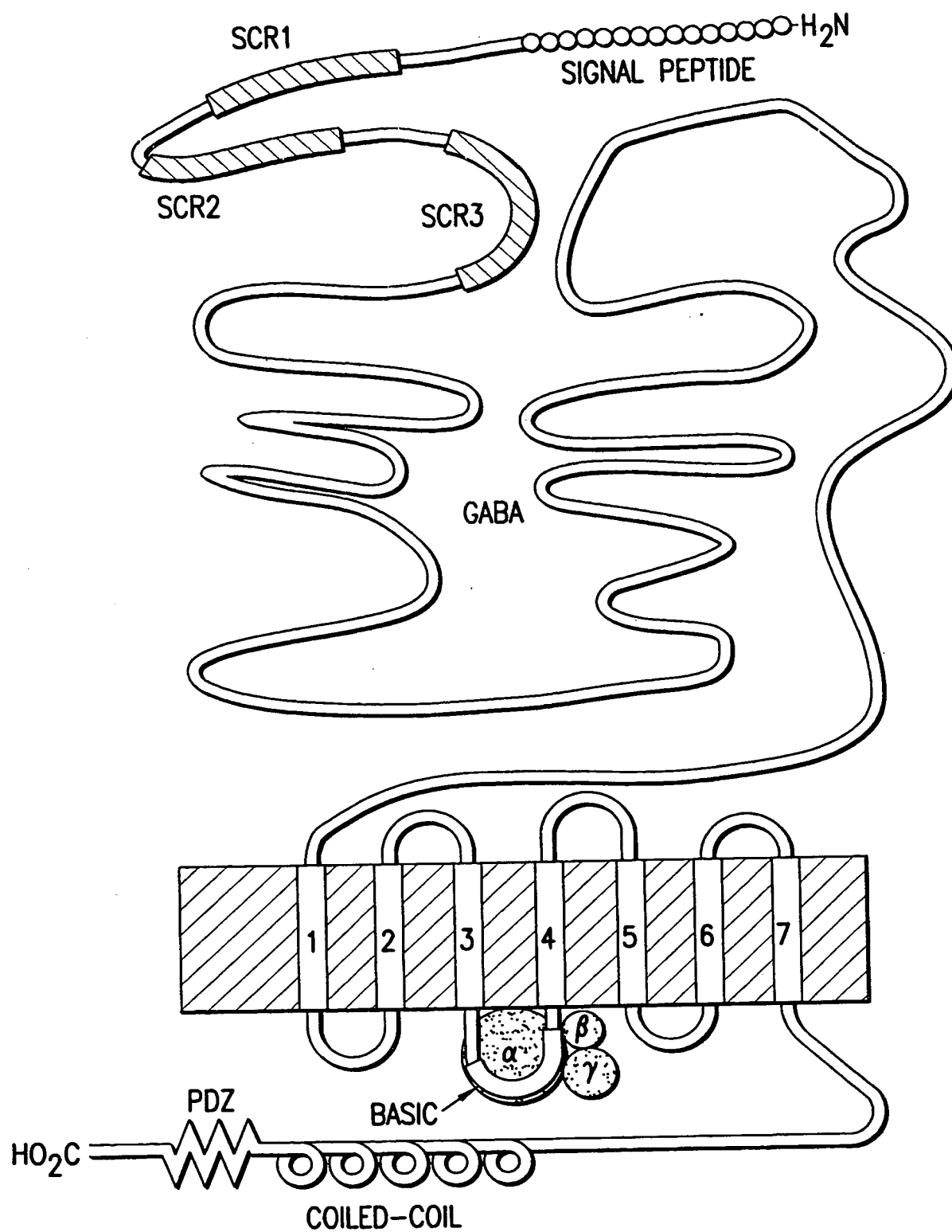


FIG.26B

SUBSTITUTE SHEET (RULE 26)

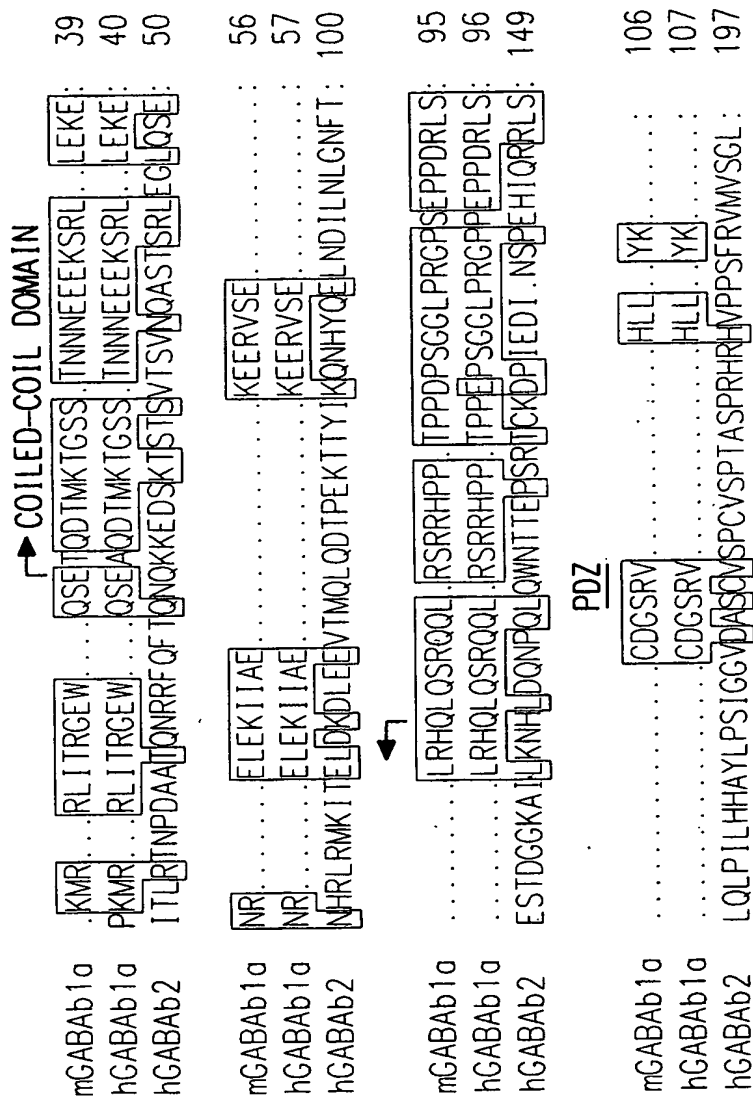


FIG.27

THIS PAGE BLANK (USPTO)